

CHEMICAL MODIFICATION OF ALPHA-AMANITIN TO
YIELD DERIVATIVES SUITABLE FOR CONJUGATION
TO PROTEINS VIA REDUCTIVE ALKYLATION

By

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LIST OF ABBREVIATIONS

ABGG.....	α -amanitiny1-7'-azobenzoylglycylglycine
ABGG-GLU.....	α -amanitiny1-7'-azobenzoylglycylglycylglucosamine
ABH.....	α -amanitiny1-7'-azobenzoyl-1,6-diaminohexane
AMA.....	α -amanitin
BSA.....	bovine serum albumin
CD.....	circular dichroism
cinn-HCl.....	<u>trans</u> -cinnamaldehyde-HCl fumes; TLC spray reagent
CT RNAP II.....	calf thymus RNA polymerase II
d ₆ -DMSO.....	perdeuterated dimethylsulfoxide
EDC.....	1-ethyl-3-(3-dimethylamino)propylcarbodiimide
EDTA.....	ethylenediaminetetraacetic acid
Fab.....	univalent antigen-binding antibody fragment
(Fab') ₂	divalent antigen-binding antibody fragment
FAB-MS.....	fast atom bombardment mass spectroscopy
HEPES.....	<u>N</u> -2-hydroxyethylpiperazine- <u>N'</u> -2-ethanesulfonic acid
HPLC.....	high-performance liquid chromatography
IT.....	immunotoxin
OMA.....	6'- <u>O</u> -methyl- α -amanitin
OMAA.....	6'- <u>O</u> -methylaldo- α -amanitin
OMA-X (X = CN, COOH, gly, NH ₂ , and pro).....	amanitin ^c derivatives defined in the text
OMDA.....	6'- <u>O</u> -methyldehydroxymethyl- α -amanitin
OML.....	6'- <u>O</u> -methylamanullin

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A toxic peptide which inhibits RNA polymerase II (RNAP II), α -amanitin, was chemically modified to generate aldehydic derivatives which were suitable for reductive coupling to proteins.

Periodate oxidation of 6'-O-methyl- α -amanitin (OMA) at neutral pH generated a mixture of two amanitin aldehydes which underwent ready interconversion. These two forms of 6'-O-methylalido- α -amanitin (OMAA) were reduced to the corresponding alcohol with sodium borohydride, but were inert to treatment with sodium chlorite at pH 2.0 or 3.0. A chemically distinct form of OMAA was produced by periodate oxidation at pH 2.0. Spectral data, in combination with the finding that this compound is easily oxidized to the corresponding carboxyl derivative by sodium chlorite at pH 2.0, indicated that this latter form of OMAA contains a free aldehyde group.

The reductive amination of OMAA with several different amines proved to be very slow, even when high reactant concentrations were

used. The products of reaction with ammonium acetate, glycine, and L-proline were all relatively poor inhibitors of calf thymus RNAP II with K_i 's of 1.7×10^{-7} M, 2.5×10^{-7} M, and 7×10^{-6} M, respectively. Conversion of the aldehyde moiety of OMAA to carboxyl and nitrile groups yielded derivatives with K_i 's of 1×10^{-7} M and 3×10^{-9} M, respectively. These data, in conjunction with those previously published, suggest that introduction of ionizable groups into this part of the amanitin molecule substantially diminishes its inhibitory potential.

An aromatic amine attached to an amino sugar via a dipeptide linker was synthesized and coupled to the 7-position of the hydroxy-tryptophan residue of α -amanitin to yield a novel azo amanitin (ABGG-GLU). Marked effects of borate, temperature, cyanoborohydride concentration, nature of the sugar residue, and pH upon the rate of conjugation of a model compound to bovine serum albumin (BSA) were delineated. Conjugation of ABGG-GLU itself to BSA was complicated by side reactions. Several combinations of reaction conditions were examined to identify ways to lower the rate of loss of ABGG-GLU to these side reactions. Raising the concentrations of BSA and ABGG-GLU provided the most effective means of achieving this goal. ABGG-GLU-BSA conjugates demonstrated K_i 's of approximately 10^{-7} M, which are comparable to those obtained by others for amanitin-BSA conjugates. The potential utility of this conjugation method is discussed.

CHAPTER I

BACKGROUND AND RATIONALE

Modified Proteins as Therapeutic Agents

Study of the chemical modification of proteins began largely as an approach to understanding the role that particular chemical moieties play in the structural integrity and function of proteins. Selective modification is still applied in this way and is an area of investigation in which significant strides continue to be made. In addition to its continued use in research activities, protein modification is currently used to produce a large variety of products. For example, proteins labeled with various enzymes and dyes are being used as very important scientific and diagnostic tools.

A relatively new area which has been under development is the application of modified proteins as therapeutic agents. The approaches to using modified proteins as therapeutic agents are legion (Sezaki and Hashida, 1984; Zaharko et al., 1979). Some simply aim at utilizing a protein as a convenient polymeric support to which a drug can be attached with the hope of a favorable alteration of the drug's pharmacokinetic properties (Hurwitz et al., 1980), but considerable attention has been focused more recently on the use of proteins which bind to specific structures in order to help a therapeutic agent to "home in" on the cell or tissue which is the desired target.

This latter avenue has been most extensively studied with the hope of developing better ways to treat neoplastic conditions, since current therapies for many cancers are associated with high levels of morbidity and mortality and often have relatively low efficacy. Antibodies that recognize tumor-associated antigens (Zalcberg and McKenzie, 1985) have come to be the preferred target-specific carriers of therapeutic agents (Ghose et al., 1983; Olsnes and Pihl, 1982; Thorpe et al., 1982), especially since the hybridoma method (Köhler and Milstein, 1975) has made antibodies of a single specificity available in almost unlimited quantities.

Conjugates of Antibodies with Protein Toxins

The development of modified antibodies as cancer therapeutic agents has proceeded primarily along two lines, conjugates made with enzymes and those made with a variety of small molecular weight cytotoxic agents (SMWCAs). The enzymatic conjugates have been prepared mainly with toxins which inactivate ribosomes and are called immunotoxins (ITs) (Blythman et al., 1981). The ribosome-inactivating proteins which have been employed to prepare ITs (reviewed in Stirpe and Barbieri, 1986) include ricin (Jansen et al., 1982; Raso, 1982; Vitetta et al., 1982), diphtheria toxin (Moolten et al., 1982), gelonin, mordeccin, abrin, pokeweed antiviral protein, and saporin (Thorpe et al., 1985a).

Although many ITs have shown potent and specific toxicity toward cells in vitro, application of these conjugates to the treatment of tumor-bearing animals has generally yielded discouraging results. As a consequence, clinical application of ITs has evolved most rapidly only

in the few circumstances where they can be used in vitro. This has mainly been limited to freeing bone marrow of unwanted populations of cells before it is transplanted (e.g., Filipovich et al., 1985).

In part because of the great promise of monoclonal antibodies and their conjugates in many therapeutic and diagnostic areas, several studies have been undertaken during the last few years in order to identify those factors which influence the localization of antibodies and their conjugates in whole animals. The important parameters which have been identified by these studies include the kinetics of conjugate access to the target cells and the kinetics of conjugate clearance. [These factors and many other issues related to the in vivo performance of antibody conjugates have been critically reviewed (Poznansky and Juliano, 1984).] From the available data, it appears that the accessibility of tumor cells to conjugates is most strongly correlated with the molecular weight of the conjugate. Smaller fragments of antibodies have more rapid access to the cells which lie in extravascular tissue spaces (Herlyn et al., 1983; Houston et al., 1980). The localization of (Fab')₂ fragments to target cells is not only more rapid but more extensive than that of whole antibody. However, the utility of very small antibody fragments, such as Fab fragments, may be limited by their loss from the circulation due to glomerular filtration (Arend and Silverblatt, 1975; Covell et al., 1986). An even more important consideration is that the conjugate must not be quickly swept from the bloodstream. Three groups of workers have recently reported their studies on the mechanism of rapid clearance of ricin and its conjugates from the circulation (Bourrie et al., 1986; Skilleter and Foxwell, 1986; Worrell et al., 1986a; Worrell et al.,

1986b). They have found that these proteins were cleared from the bloodstream in minutes by hepatic nonparenchymal cells which recognize the mannose-containing oligosaccharides borne by ricin. This has prompted efforts to modify ricin either enzymatically (Foxwell et al., 1985) or chemically (Skilleter et al., 1985; Thorpe et al., 1985b) in order to abrogate this unwanted problem. Pokeweed antiviral protein is not a glycoprotein (Stirpe and Barbieri, 1986) and so would not be expected to be cleared from the circulation by the same sort of mechanism as ricin. In fact, Ramakrishnan and Houston (1985) have reported circulatory half-lives for pokeweed antiviral protein-antibody conjugates of approximately 24 hours.

Conjugates of Antibodies with Small Molecular Weight Cytotoxic Agents

The other major approach to the production of novel cancer therapeutic agents by means of modification of antibodies has utilized SMWCAs. In most instances, investigators have employed chemotherapeutic drugs which are already being applied to the treatment of cancer. Thus, the antibody moiety is intended to provide additional specificity of action to a drug which already has some specific antitumor activity. Drugs which have been commonly employed include methotrexate (Garnett et al., 1983; Kanellos et al., 1985; Kulkarni et al., 1981), daunorubicin (Arnon and Sela, 1982; Shen and Ryser, 1981), vindesine (Ford et al., 1983), radioisotopes (Badger et al., 1985; Meares et al., 1984), and various alkylating agents (de Weger et al., 1982). These conjugates have typically had much lower potency in vitro than those prepared with the protein toxins. Despite this, experiments with animals and small clinical trials with cancer patients have, in some

cases, been quite encouraging.

Some of the same factors outlined above (e.g., size of the conjugate) which modulate the efficacy of the protein toxin conjugates in vivo, may also influence the efficacy of the SMWCA conjugates. In addition, there may be other mechanisms by which SMWCA conjugates are cleared rapidly from the circulation by the reticuloendothelial system. This will be discussed in greater detail below.

Immunotoxins Versus Conjugates Made with Small Molecular Weight Cytotoxic Agents

As of yet, a clear choice between ITs and SMWCA conjugates as the superior alternative cannot be made. Each type of conjugate has its own distinct advantages and disadvantages. Because of the extremely high potency of the enzymatic toxins (Yamaizumi et al., 1978), ITs need only contain a single molecule of toxin per antibody molecule. Consequently, any deleterious effect on the antigen-binding activity of the antibody molecule may be small. On the other hand, the low molecular potency of the SMWCAs makes it desirable to prepare conjugates with as many SMWCA molecules linked to each antibody molecule as can be tolerated. Unfortunately, difficulties with low solubility of the conjugates and loss of antigen-binding activity often intervene at relatively low levels of conjugation. For example, antibody conjugates prepared with methotrexate have been reported to lose solubility and antigen-binding activity with greater than ten (Kulkarni et al., 1981) or twelve (Kanellos et al., 1985) molecules of methotrexate per antibody molecule. Ford et al. (1983) indicated that antibody conjugates with more than eleven molecules of vindesine became poorly soluble. Some workers have sought to circumvent this problem by linking the

SMWCA first to a highly soluble carrier and then coupling the loaded carrier to an antibody. For example, Garnett et al. (1983) conjugated an average of 32 molecules of methotrexate to each molecule of human serum albumin. This drug-albumin conjugate was then linked to a monoclonal antibody. Another group of workers has used poly-L-glutamic acid as an intermediate carrier for daunorubicin (Kato et al., 1984; Tsukada et al., 1984). An advantage of the SMWCAs is the relative insensitivity of the SMWCAs to lysosomal hydrolases, while the protein toxins are apparently very susceptible to proteolytic digestion in lysosomes. Ammonium chloride has been found to greatly increase the potency of ITs, presumably by suppressing the function of lysosomes in the target cells (e.g., Jansen et al., 1982). Also in their favor, the conjugated SMWCAs need not greatly increase the molecular weight of the antibody, while the protein toxins generally have significant molecular weights (greater than 30,000 daltons). As already discussed above, the higher molecular weight may have a significant negative impact upon the rate at which the conjugate can reach the tumor from the intravascular space.

Conjugates of Amatoxins with Proteins

Conjugation of amatoxins to proteins, which is the subject of this work, has been studied in several laboratories (reviewed in Faulstich and Fiume, 1985). Amatoxins are the constituents of certain mushrooms which cause the vast majority of fatal mushroom poisonings. Work by Wieland and his colleagues has revealed the amatoxins to be cyclic octapeptides which have several unusual amino acid residues (Fig. I-1). These peptides exert their toxic action by inhibiting the

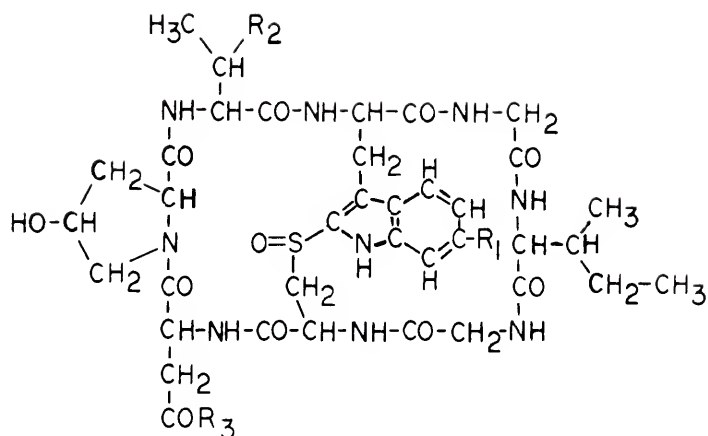


Fig. I-1. Structures of some pertinent amanitins.

<u>amanitin</u>	<u>R₁</u>	<u>R₂</u>	<u>R₃</u>
α-amanitin	OH	CHOHCH ₂ OH	NH ₂
β-amanitin	OH	CHOHCH ₂ OH	OH
γ-amanitin	OH	CHOHCH ₃	NH ₂
amanullin	OH	CH ₂ CH ₃	NH ₂
OML	OCH ₃	CH ₂ CH ₃	NH ₂
OMDA	OCH ₃	CH ₂ OH	NH ₂
OMA	OCH ₃	CHOHCH ₂ OH	NH ₂
OMAA	OCH ₃	CHO	NH ₂

DNA-dependent RNA polymerases which are responsible for transcription of nuclear genes. The inhibition of the class II enzyme, which generates messenger RNA, is particularly potent with a K_i of approximately 10^{-9} M (reviewed in Wieland, 1983).

The first reports concerning amatoxin-protein conjugates arose from efforts to prepare amatoxin-specific antisera. An amatoxin with a free carboxyl group, β -amanitin, was linked to bovine serum albumin (BSA) with the aid of a water-soluble carbodiimide. When administered to rabbits, these conjugates proved to be much more toxic than the free toxin (Bonetti et al., 1976; Cessi and Fiume, 1969). Subsequent pathological studies showed that this increased toxicity was due to uptake of the conjugate by Kupffer cells (Derenzini et al., 1973). Thus, this represented accidental targeting of the toxin into the reticuloendothelial system by virtue of its conjugation to BSA. An increase in toxicity of this conjugate relative to free toxin was also demonstrated for macrophages in culture (Barbanti-Brodano and Fiume, 1973; Fiume and Barbanti-Brodano, 1974).

A number of conjugates have also been prepared with azo derivatives of α -amanitin (Faulstich and Trischmann, 1973; Preston et al., 1981) which have been referred to as ABH and ABGG (short for α -amanitinyl-7'-azobenzoyl-1,6-diaminohexane and α -amanitinyl-7'-azobenzoylglycylglycine; previously called ADH and ADGG). Both of these amatoxin derivatives have been coupled to proteins with the aid of a water-soluble carbodiimide. When the toxicity of conjugates made from BSA and ABH was tested on several cell lines, a positive correlation between toxicity and phagocytic rate was found (Hencin and Preston, 1979). Conjugates prepared with the lectin concanavalin A proved to be

potent cytotoxins, the toxicity of which could be greatly diminished by the addition of certain sugars which are bound by the lectin and thus inhibit binding to the cell surface (Hencin, 1979). Linkage of ABGG to a monoclonal antibody which recognizes Thy 1.2, an antigenic marker of mouse T cells, yielded a conjugate with potent, specific toxicity in vitro toward cells which bear the Thy 1.2 antigen (Davis and Preston, 1981).

Because of certain characteristics of amatoxins, the amatoxin-antibody conjugates fall within a class which is distinct from either ITs or SMWCA-antibody conjugates. Amatoxins are like the ribosome-inactivating enzymes used in ITs in that they are nonspecific cytotoxins, but they are like the SMWCAs in that they have a relatively small molecular weight and resistance to hydrolases. Someday the amatoxin-antibody conjugates may prove to be useful as therapeutic agents and, as members of a distinct class, they may be uniquely suited to some applications.

The amatoxins share with the SMWCAs a relatively low molecular potency compared to the ribosome-inactivating toxins. Thus, one can anticipate the need for relatively high levels of conjugation to antibody, especially in systems where there are few target antigenic sites per cell. However, for amatoxin-antibody conjugates the importance of retaining antigen-binding activity and favorable pharmacokinetic behavior (i.e., long circulatory half-life and rapid penetration to target cells) is more critical than for the conjugates made with the chemotherapeutic drugs. This is true because the drugs possess considerable selective toxicity toward many types of neoplastic cells without being conjugated to an antibody, while amatoxins do not. Amatoxin-

protein conjugates can potentially kill any cell which takes them up. These considerations have prompted a search for a mode of conjugation of SMWCAs (as exemplified by amanitin) to antibodies which would represent a theoretical optimum.

Toward Optimal Conjugation of Small Molecular Weight Cytotoxic Agents to Proteins

Because of the wide utility of chemically modified antibodies, a number of investigators have compared the effect of different kinds of modifications on the ability of the antibodies to retain their antigen-binding activity. Most of the currently employed conjugation methods rely upon reaction with the amino groups of the antibody. This is because many of the amino groups are disposed toward the surface of the protein, they are reactive as nucleophiles at neutral to mildly alkaline pH's, and they can be modified with high selectivity with several classes of reagent.

A reasonable premise might be that for the conjugation of any given chemical entity (e.g., SMWCA) to an antibody, a method which preserves the native charges of the antibody should produce the least possible alteration in its structure. In fact, modifications which preserve the positive charge of the antibody amino groups, including reductive alkylation and amidination, generally have a lesser effect on antigen-binding activity than those modifications which abolish the charge of the antibody amino groups (see below). Both amidination and reductive alkylation are reactions which are highly specific for amino groups of proteins. The utility of amidination is somewhat limited by the harsh conditions required for generating the imidate esters which are the amidinating reagents. On the other hand, reductive alkylation

with sodium cyanoborohydride (Borch et al., 1971) and an aldehyde has received increasing attention as an excellent method for specifically and gently modifying proteins (Hutchins and Natale, 1979).

Cohen and Becker (1968) have shown that the precipitin activity of several different anti-hapten antibodies was greatly inhibited by low levels of carbamylation, while it was preserved at high levels of amidination with ethyl acetimidate. The carbamyl and amidino groups are similar in size and shape; they differ principally in that the amidino group bears a positive charge, while the carbamyl group is uncharged. Thus, this study demonstrated especially well the importance of preserving the native charges of the antibody. Studies of this kind have prompted the introduction of amidinating reagents for the haptenation of antibodies to be used in hapten-sandwich techniques. Wofsy and his colleagues (1978) have synthesized azo haptens which can be coupled to antibodies at levels greater than thirty hapten moieties per antibody molecule with good preservation of the antigen-binding activity. A similar reagent for introducing the dinitrophenyl group has recently been reported which can be used to prepare conjugates with up to thirteen dinitrophenyl groups per antibody molecule without loss of antigen-binding activity (Hewlins et al., 1984).

Radiolabeling of antibodies by reductive methylation with tritium-labeled sodium borohydride and formaldehyde has gained in popularity because of the high degree of preservation of antigen-binding activity which is achieved with this method (Tack and Wilder, 1981). Reductive alkylation has, however, not been frequently used to attach larger chemical moieties to antibodies despite the good results which have been obtained with reductive methylation.

In addition to deleterious effects on antigen-binding activity, chemical modifications of antibodies which remove positive charges may also depress their circulatory half-life. Several kinds of chemical modification have been found to promote clearance of proteins from the circulation of mammals. These modifications include acylation, reaction with formaldehyde, and dinitrophenylation. Much of the work on acylated proteins has focused on lipoproteins (Brown et al., 1980). Cells of the reticuloendothelial system bear specific, high-affinity receptors for certain acylated proteins (Mahley et al., 1980; Nagelkerke et al., 1983; Pitas et al., 1985). Formaldehyde-modified albumin is taken up by the same cells, but apparently by a separate receptor (Blomhoff et al., 1984; Horiuchi et al., 1985; Horiuchi et al., 1986). Dinitrophenylated albumin has also been found to be cleared by the reticuloendothelial system, primarily in the liver (Kitteringham et al., 1985; Rhodes and Aasted, 1973; Skogh, 1982; Skogh et al., 1983). These three classes of chemical modification each reduce the number of native positive charges on the proteins and thus make them more anionic. In the case of the receptor for acylated lipoproteins it has been specifically shown that certain polyanions can compete for binding to the receptor (Mahley et al., 1980). This suggests that the anionic character of the modified protein contributes to its affinity for this receptor. Two groups of workers have observed a correlation between increasing localization to the liver of antibodies coupled with diethylenetriaminepentaacetic acid, a metal chelator, and increasing levels of conjugation (Anderson and Strand, 1985; Sakahara et al., 1985). In these studies, the chelator was linked to the antibodies by an acylation reaction. Winkelhake (1977)

has also noted substantial decreases in the circulatory half-life of an acylated antibody, while reductively methylated antibody was cleared much more slowly.

Unfortunately, there is relatively little information concerning the fate of SMWCA-antibody conjugates in vivo. The information obtained in other systems, as described above, suggests that the most frequently used method of preparing SMWCA-antibody conjugates (i.e., acylation) may not be optimal. The available data also suggest that a conjugation method which preserves the native charges of the antibody, such as amidination or reductive alkylation, may provide better retention of antigen-binding activity and longer circulatory half-life.

Rationale for the Current Work

The studies described below have been directed toward the development of a practical method by which a suitable amatoxin derivative can be conjugated to proteins by means of reductive alkylation. Development of a method of this kind would provide a future opportunity to examine its usefulness relative to existent methods for conjugation of amatoxins to antibodies, especially with regard to any effects on antigen-binding activity and circulatory half-life in animals.

Preparation of an amatoxin useful for reductive alkylation requires the introduction of an aldehyde group into the molecule. Fig. I-2 shows the sites at which α -amanitin (AMA), the most readily available amatoxin, can be easily and selectively modified. The dihydroxyisoleucine residue (site A) is susceptible to oxidation by periodate to yield an aldehyde (Wieland and Fahrmeir, 1970). The 7-position of the hydroxytryptophan residue (site B) is subject to

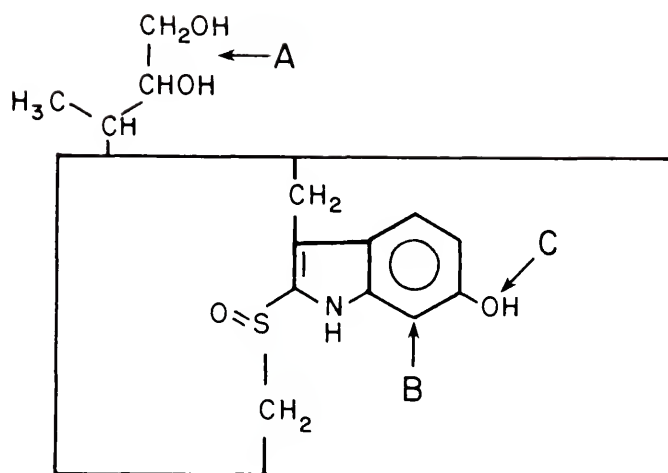


Fig. I-2. Sites for selective chemical modification of α -amanitin.

electrophilic substitution (Faulstich and Trischmann, 1973; Morris and Venton, 1983). For example, relatively complex aromatic amines can be diazotized and reacted with AMA to yield azo derivatives like ABGG. Also, the hydroxyl group of the hydroxytryptophan moiety (site C) has been alkylated with alkyl halides to prepare useful derivatives (Faulstich et al., 1981).

Two of these three possible sites were chosen for evaluation. Since periodate oxidation of the dihydroxyisoleucine residue yields an aldehyde function in high yield, the feasibility of using this approach was examined. Also, since the azo derivative ABGG had already been utilized to synthesize several potent conjugates, a derivative of ABGG which contained an aldehyde function was prepared and studied.

CHAPTER II

STUDIES ON THE NATURE OF 6'-O-METHYLALDO- α -AMANITIN

Introduction

The oxidation of 6'-O-methyl- α -amanitin (OMA) with sodium periodate to yield 6'-O-methylaldo- α -amanitin (OMAA) was first reported in 1970 (Wieland and Fahrmeir). Like amanullin (Fig. I-1), OMAA was demonstrated to be nontoxic when injected into mice. However, when the aldehyde function of OMAA was reduced with sodium borohydride to a hydroxyl group, the resultant derivative, 6'-O-methyldehydroxymethyl- α -amanitin (OMDA), proved to be toxic to mice. These and other data prompted the hypothesis that the γ -hydroxyl of the dihydroxyisoleucine (residue 3) sidechain was critical for the toxic action of amatoxins (Wieland and Fahrmeir, 1970).

After it was learned that the amatoxins express their toxicity by means of potent inhibition of RNA polymerase II (RNAP II), studies showed that OMAA was a very poor inhibitor of RNAP II (Buku et al., 1971), while amanullin was found to be a potent inhibitor (Cochet-Meilhac and Chambon, 1974). The low toxicity of amanullin in mice has been rationalized in terms of unusual pharmacokinetic properties. A possible explanation for the low inhibitory activity of OMAA toward RNAP II was found when its circular dichroism (CD) spectrum was discovered to be greatly different from that of other amanitins. This alteration in the CD spectrum and attendant loss of toxicity were

interpreted as being due to a change in the conformation of the peptide backbone. It was proposed that the conformational change was induced by the formation of an intramolecular hydrogen bond to the oxygen of the aldehyde (Faulstich et al., 1973). Further support for an altered conformation of OMAA has come from radioimmunoassay studies. Faulstich and Wieland (1975) found that more than 120 times as much OMAA as compared to AMA was required to displace half of the radiolabeled amatoxin from amatoxin-specific antibodies.

Unpublished work by Dr. James F. Preston has suggested that there are actually at least two products which result from the periodate oxidation of OMA and which can be chromatographically separated on a column of Sephadex LH-20 eluted with water. These oxidation products demonstrated the same mobility on TLC and reacted with trans-cinnamaldehyde and HCl (cinn-HCl) to yield the rust color which is distinctive of OMAA (Wieland and Fahrmeir, 1970). Each one also was reduced by sodium borohydride to a compound with the characteristics of OMDA, including violet color reaction with cinn-HCl and potent inhibition of calf thymus RNA polymerase II (CT RNAP II).

The nature of these two periodate oxidation products was reevaluated here in order to determine whether one or both components should be used in reductive alkylation studies and to obtain insights into their chemistry which might be pertinent to the intended conjugation to protein amino groups.

Materials and Methods

Materials

Reagents. All chemicals were analytical reagent grade unless

otherwise specified. *p*-Tolylsulphonylmethylnitrosamide and tetramethylsilane (TMS) were obtained from Aldrich Chemical; sodium periodate, sodium borohydride, D-glucose, and d_6 -dimethylsulfoxide (d_6 -DMSO) from Sigma Chemical; and sodium chlorite and amidosulfonic acid from Alfa Chemical. Solvents and trifluoroacetic acid (TFA) were obtained from Fisher Scientific. Water used in the experiments described in this and subsequent chapters was deionized and then distilled from glass.

Toxin. The amatoxin AMA was purified from carpophores of Amanita suballiacea (Murr.) Murr. collected in the Gainesville, Florida, area by a modification of methods previously described (Little and Preston, 1984). Briefly, the toxin was obtained as follows: Coarsely chopped carpophores (800 g) were extracted with 1.2 l of methanol for approximately 24 h. The filtered and concentrated crude toxin solution was extracted three times with three volumes of diethyl ether. The defatted aqueous phase was then mixed with nine volumes of methanol and refrigerated overnight. This was then filtered to remove the precipitated polar compounds (primarily carbohydrates and salts). After concentration the toxin was partially purified by column chromatography on Sephadex LH-20 eluting with 50% methanol, on Bio-gel P-2 eluting with water, and on Sephadex LH-20 eluting with water. The remaining impurities were removed by high performance liquid chromatography (HPLC) on a Zorbax ODS column (0.94 X 25 cm, Du Pont) eluting with 15% acetonitrile and/or by recrystallization from methanol.

Estimates of the concentration of aqueous amanitin solutions were based upon an extinction coefficient at 304 nm of $15,400 \text{ M}^{-1} \text{ cm}^{-1}$ (cf. Cochet-Meilhac and Chambon, 1974). Because of the pH-dependence of its

absorbance spectrum, measurements of absorbance at 304 nm of AMA were made in 5 mM sodium phosphate buffer, pH 7.0. Yields of amanitin derivatives were calculated from these estimates of concentration and the volume of the solutions.

Analytical Procedures

Spectroscopy. Absorbance measurements were made with the aid of a Gilford 2400 spectrophotometer. Absorbance spectra in both the ultra-violet (UV) and visible ranges were obtained on either a Beckman 25 spectrophotometer or a Hewlett-Packard 8451A diode array spectrophotometer. Circular dichroism (CD) spectra were recorded on a Jasco J-500C spectropolarimeter with the aid of Jasco IF-500 and Okidata IF-800 data processing equipment. Proton magnetic resonance (PMR) spectra were obtained on a Nicolet NT-300 spectrometer operating at 300 MHz in the Fourier transform mode.

Chromatography. Thin-layer chromatography (TLC) was performed on 0.25 mm layers of silica gel 60 F254 (Merck). TLC solvent system I contained 1-butanol: acetic acid: water (4:1:1). Amatoxins were detected on chromatograms by means of their quenching of the layer's fluorescence and by their color reaction after being sprayed with 2% methanolic trans-cinnamaldehyde and subsequent exposure to HCl fumes (cinn-HCl). HPLC was performed with a Waters model 6000A pump which was equipped with a U6K injector. Elution of compounds was detected by a Gilson Holochrome variable wavelength absorbance monitor. Samples were chromatographed on a Zorbax ODS column (0.94 X 25 cm, Du Pont) which was protected by a 0.5 μ m prefilter (Rainin) and a C5 guard cartridge (Bio-Rad). Samples for HPLC were filtered through 0.22 μ m

Millex GV or SR filters (Millipore).

RNA polymerase assay. Calf thymus RNA polymerase II (CT RNAP II) was prepared and stored as previously described (Preston et al., 1975). Components of the reaction mixture were those of Cochet-Meilhac and Chambon (1974) except that [^3H]UTP was employed for labeling the product. The assay was performed as follows: The reaction tube with 10 μl of amanitin solution was incubated at 37°C for 10 min. The enzyme solution was added to this and allowed to incubate for 10 min more at 37°C. The reaction was started by adding a solution containing the nucleoside triphosphates to yield a final reaction volume of 100 μl . After 10 min at 37°C the reaction was stopped and processed for scintillation counting as previously described (Preston et al., 1975). Counting was performed on Beckman LS-133 and LS-8000 liquid scintillation counters. Inhibition data were analyzed according to Dixon (1953). The best fit of lines to data points was estimated by the method of least squares using the statistical functions of a Texas Instruments TI-55-II calculator.

Synthesis of Amanitin Derivatives

Synthesis of 6'-O-methyl- α -amanitin. To 30 μmol of AMA in 10.5 ml of ice-cold methanol were added 4.5 ml of an ice-cold ethereal-ethanolic solution (de Boer and Backer, 1954) of diazomethane which was generated by the action potassium hydroxide on *p*-tolylsulphonylmethylnitrosamide and adjusted to 0.10 M with ice-cold ether. The diazomethane solution was titrated according to Arndt (1943) prior to use. (Note: Diazomethane is a toxic and potentially explosive gas. It should only be prepared and used in a well-ventilated hood, preferably

behind a safety shield, in glassware which lacks ground or abraded surfaces.) The reaction mixture was permitted to rise to room temperature in a water bath in the dark. After 30 min the remaining diazomethane and the bulk of the ether were evaporated under a stream of nitrogen. The resultant solution was concentrated to dryness in a rotary evaporator at 30°C, redissolved in water, filtered, adjusted to 22% acetonitrile, and subjected to HPLC with the same solvent mixture. The desired product eluted between 24 and 32 ml. The yield was 14.6 μmol (49%); 14.4 μmol (48%) of the AMA was recovered unchanged. TLC: R_F , 1-0.47; color-reaction, violet.

Synthesis of 6'-O-methylaldo- α -amanitin under neutral conditions.

To 20.3 μmol of OMA in 1.0 ml of water was added 0.5 ml of a solution containing 22.0 μmol of sodium periodate. After 5 min of reaction in the dark at room temperature residual sodium periodate was quenched by the addition of 10 μl of ethylene glycol. The solution was filtered, adjusted to 22% with respect to acetonitrile, and subjected to HPLC eluting with 22% acetonitrile. The desired products eluted between 28 and 45 ml. The yield was 18.8 μmol (92%). TLC: R_F , 1-0.43; color-reaction, rust-red.

Synthesis of 6'-O-methyldehydroxymethyl- α -amanitin. OMA (2.0 μmol) was oxidized with periodate as described above. The components of the reaction mixture were separated by HPLC eluting with 18% acetonitrile in order to achieve complete separation of the products. The resultant fractions were placed on ice as they were collected. Under these chromatographic conditions the products OMAA-IA and OMAA-IB eluted between 57 and 67 ml and between 85 and 95 ml, respectively. To 0.20 μmol each of OMAA-IA and OMAA-IB was added 1.0 μmol of

sodium borohydride in 80 μ l of water. After gentle mixing, this was permitted to react for an hour in the dark at 23°C. Residual sodium borohydride was quenched by reaction with 10 μ l of 1.0 M D-glucose for a further hour under the same conditions. The reaction mixture was rinsed from the vial with 1.0 ml of 22% acetonitrile and then chromatographed by HPLC using the same solvent. A single product eluted between 29 and 34 ml in 75-90% yield. TLC: R_F , I-0.51; color-reaction, violet.

Synthesis of 6'-O-methylaldo- α -amanitin under acidic conditions.

To OMA (8 μ mol) in 0.75 ml of 50 mM sodium phosphate, pH 3.0, was added sodium periodate (8.8 μ mol) in 30 μ l of water. After reaction for 5 min at 23°C in the dark, the solution was adjusted to 22% with respect to acetonitrile and applied to HPLC eluting at 1 ml/min with acetonitrile: 0.05% TFA (22:78). The desired product (7.0 μ mol, 88%) eluted between 30 and 35 ml. TLC: R_F , I-0.61; color-reaction, violet.

One-half of this material was prepared for PMR studies. After the acetonitrile was removed at 30°C in vacuo, the sample was lyophilized and redissolved in 1.0 ml of d_6 -DMSO.

Results

Synthesis of 6'-O-methyl- α -amanitin

The synthesis of OMA has been patterned after that of Wieland and Fahrmeir (1970). The major disadvantage of this synthetic route is the use of the dangerous diazomethane; alternatively, AMA can be methylated with methyl iodide (Faulstich et al., 1981). Studies on the synthesis of OMA using diazomethane (Little, 1984) have demonstrated

that the choice of reaction conditions is quite critical. Both reactant stoichiometries and concentrations are important. At stoichiometries and/or concentrations less than the optimum the yield of OMA falls rapidly, but virtually quantitative recovery of input toxin is achieved as the sum of AMA and OMA. At stoichiometries and/or concentrations greater than the optimum the yield of OMA falls because of the production of byproducts, one of which has been identified as 1',6'-N,O-dimethyl- α -amanitin (Little, 1984). The conditions described here are similar to those of Little (1984) and provide for recovery of virtually all of the input toxin as the sum of OMA and recovered AMA.

OMA shares many characteristics in common with AMA, including potent inhibition of CT RNAP II, UV absorbance spectrum, and violet color reaction with trans-cinnamaldehyde. However, it can be readily distinguished from AMA on the basis of a distinct CD spectrum and the resistance of its UV absorbance spectrum to bathochromic shift upon alkalinization (Faulstich et al., 1973; Faulstich et al., 1981).

Products of Periodate Oxidation of 6'-O-methyl- α -amanitin under Conditions of Neutral pH

HPLC purification of the periodate oxidation products. Separation of the products of periodate oxidation of OMA by reverse-phase HPLC yielded two components, just as was seen previously when the separation was performed by conventional chromatography. These two entities will be referred to as OMAA-IA for the material eluting first and OMAA-IB for the material which eluted later (Fig. II-1). When the HPLC fractions were held at room temperature, reanalysis at intervals of the purified components by HPLC demonstrated a slow interconversion of the two forms (Fig. II-2). Within 2 h OMAA-IA (eluting at

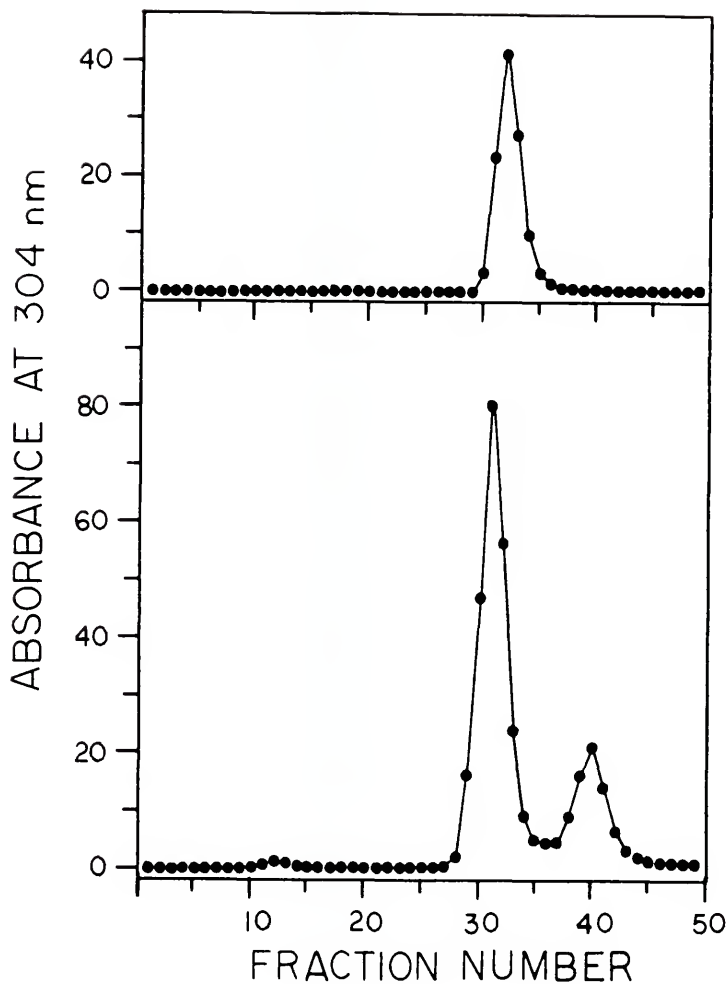


Fig. II-1. Preparative HPLC purification of various forms of OMAA on a reverse-phase C18 column. The upper panel shows the purification of OMAA-II with acetonitrile: 0.05% TFA (22:78) as mobile phase. The lower panel shows the purification of OMAA-I with 22% acetonitrile as the mobile phase. Fractions contained 1.0 ml.

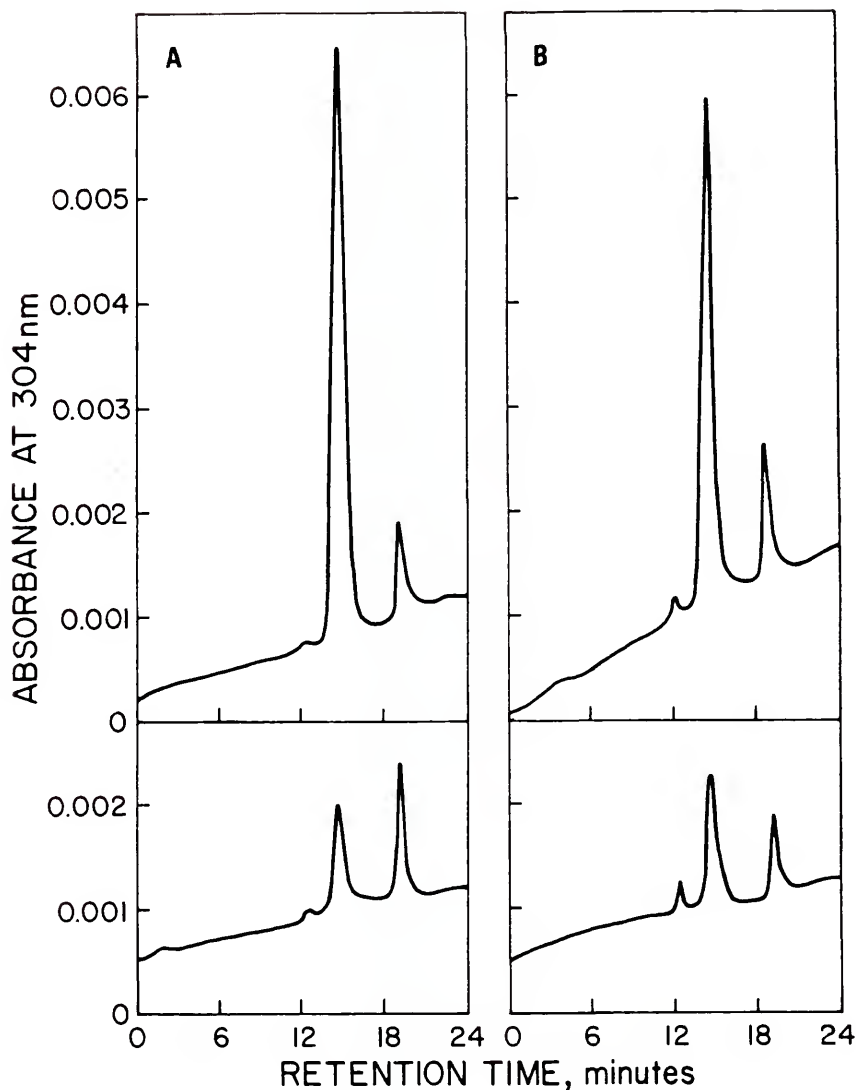


Fig. II-2. HPLC analysis of components of OMAA-I. Samples (30 μ l) of OMAA-IA (upper tracings) and OMAA-IB (lower tracings) were rechromatographed on a reverse-phase C18 column with 22% acetonitrile at 2 ml/min at 2 h (panel A) and 26 h (panel B) after their initial purification by preparative HPLC.

about 14 min) was contaminated with significant amounts of OMAA-IB which eluted at about 19 min (upper portion of panel A) and after 26 h further conversion was apparent (upper portion of panel B). An even more dramatic conversion of OMAA-IB to OMAA-IA was observed (lower portion of panels A and B).

Spectral and TLC properties of the periodate oxidation products.

The two OMAA-I components have UV absorbance spectra which are indistinguishable from each other and very similar to that of OMA. When held on ice, the interchange between the two products described above was retarded. Indeed, when HPLC fractions which derived from separation of the periodate oxidation products of OMA were placed on ice immediately after they were collected, no contamination of one form with the other could be detected by HPLC analyses repeated over 48 hours. This aided in the acquisition of CD spectra of the two products in their pure forms. Thus, HPLC fractions were placed on ice as they were collected and warmed to room temperature just before the CD spectrum was obtained. HPLC analysis also showed that fractions did not undergo significant transformation to the other form during the time at room temperature which was needed for acquisition of the CD spectrum. The CD spectra of the periodate oxidation products were indistinguishable from each other and from that previously published for OMAA (Fig. II-3).

A PMR spectrum of OMAA-I dissolved in d_6 -DMSO showed only a very small peak in the region expected to contain the signal of an aldehydic proton ($\delta=9.5-10$). In addition, the spectrum lacked the sharp definition of peaks which has been characteristic of the PMR spectra of other amanitin derivatives (data not shown).

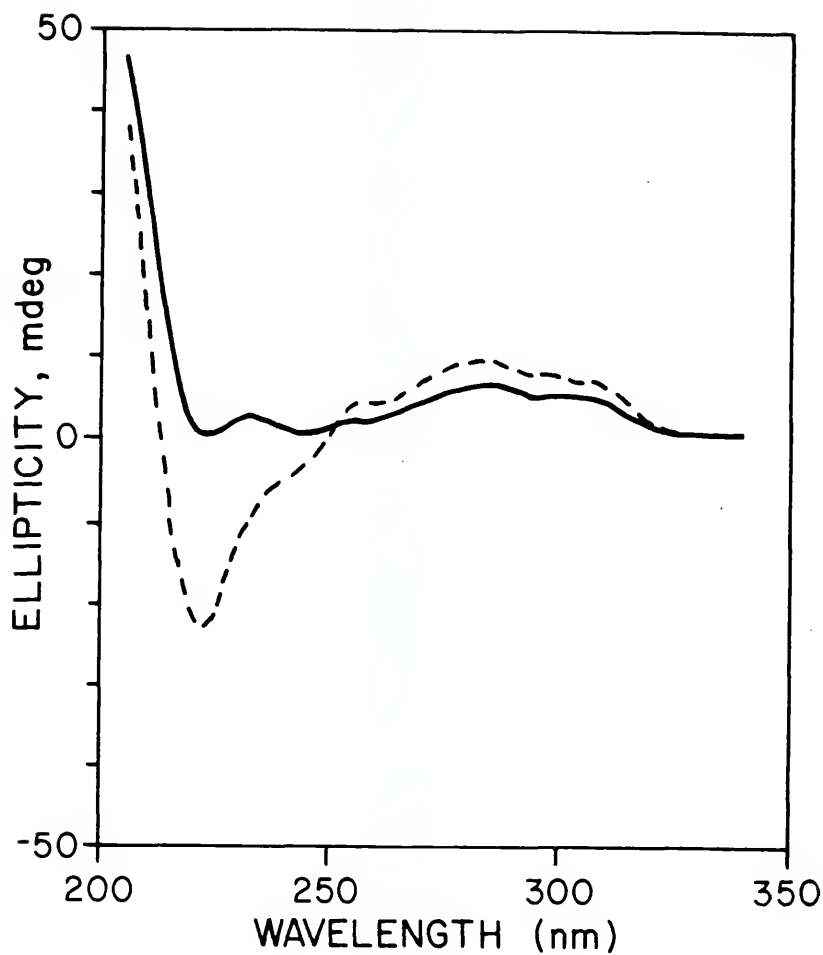


Fig. II-3. CD spectra of OMA (dashed line) and OMAA-I (solid line) in water at concentrations of approximately 4×10^{-5} M.

Chemical reactivity of the periodate oxidation products.

Reduction of OMAA-IA and OMAA-IB with sodium borohydride produced in 75-90% yield a toxin derivative with the properties expected of OMDA. The reduction products from these forms of OMAA were indistinguishable in terms of UV absorbance and CD spectra (which were identical to those of OMA), TLC and HPLC mobility, violet color reaction with cinn-HCl, and inhibition of CT RNAP II with K_i of $3.4 \pm 0.2 \times 10^{-9}$ M. In contrast to their ready reduction with sodium borohydride, neither OMAA-IA nor OMAA-IB was susceptible to the action of the oxidant sodium chlorite at pH 2.0 or 3.0 (Launer and Tomimatsu, 1954; Lindgren and Nilsson, 1973).

Product of Periodate Oxidation of 6'-O-methyl- α -amanitin at pH 3.0

HPLC purification of the periodate oxidation product. In contrast to the reaction at neutral pH, periodate oxidation of OMA at mildly acidic pH's yielded only a single product which was distinct from the periodate oxidation products described above and which could be purified by reverse-phase HPLC using an acidic mobile phase (Fig. II-1). This form of OMAA will be referred to as OMAA-II.

Spectral and TLC properties of the periodate oxidation product.

The material prepared and purified under acidic conditions had many properties which were strikingly different from those of OMAA-IA and OMAA-IB. TLC analysis of HPLC fractions (or of the reaction mixture) showed predominantly a single component with higher mobility which stained violet with cinn-HCl; there was trace contamination with the rust-staining OMAA-I forms. TLC analysis of these same HPLC fractions in solvent system I after they were neutralized with sodium phosphate

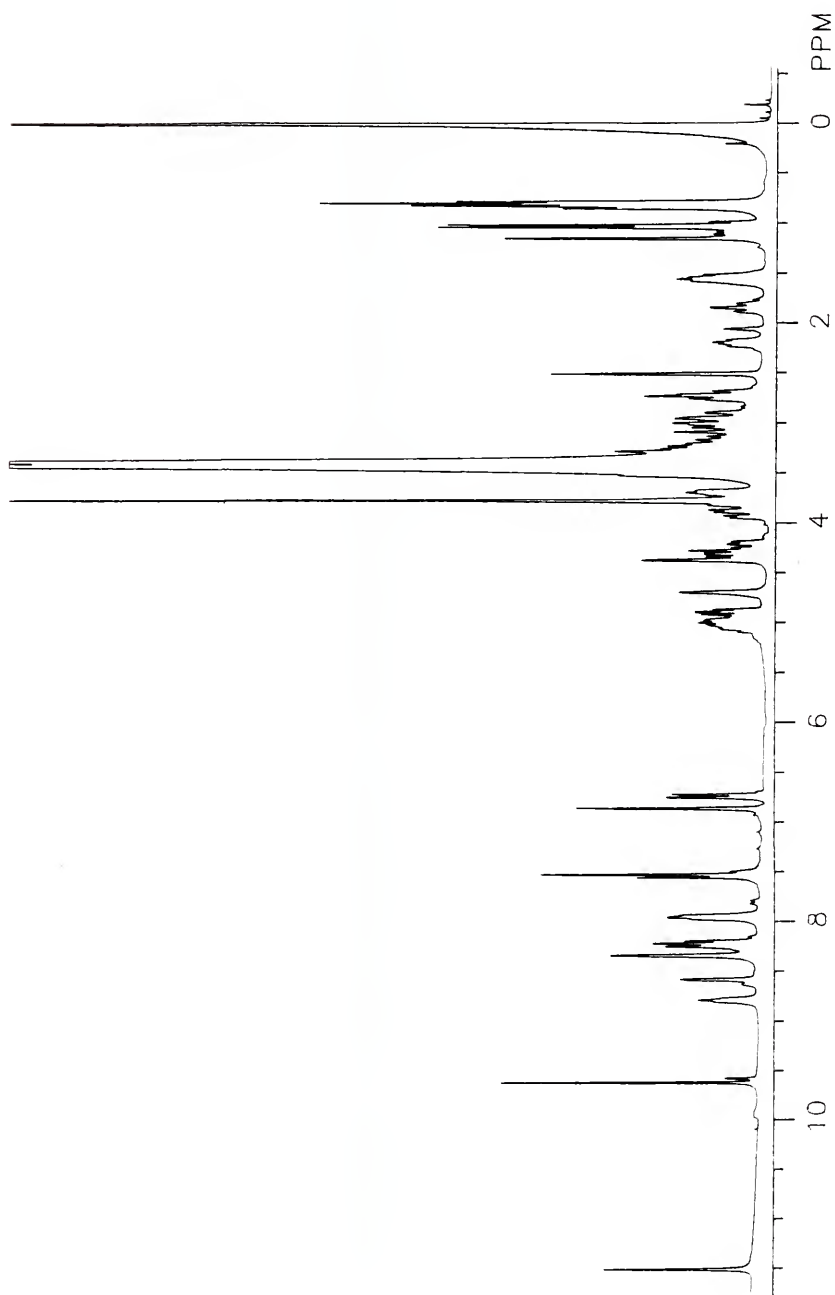
buffer and immediately spotted onto a TLC plate showed primarily the rust-staining OMAA-I and a trace of the violet-staining OMAA-II. The UV absorbance and CD spectra of OMAA-II were indistinguishable from those of OMA. However, within thirty minutes of its neutralization with sodium phosphate buffer the CD spectrum of OMAA-II changed to that typical of OMAA-IA and OMAA-IB. A PMR spectrum of OMAA-II in d_6 -DMSO showed the general features expected of a methylaminitin (Wieland et al., 1983) and also a peak of appropriate size at $\delta=9.55$ as expected for an aldehyde (Fig. II-4).

Chemical reactivity of the periodate oxidation product. When both sodium chlorite and sodium periodate were added to a solution of OMA which was buffered at pH 2.0 or 3.0, a novel product was generated. This product has been identified as the carboxylic acid (OMA-COOH) which results from the oxidation of the aldehyde function of OMAA (see chap. III).

Discussion

The chemical nature of OMAA is of interest for at least two reasons. First, since OMAA-I has an altered conformation, detailed information about its nature can provide some insight into the forces which maintain amatoxins in their toxic conformation. Second, knowledge of its chemistry provides a sound base upon which the planning of semisynthetic modifications to this part of the toxin can rest. It is perhaps because the chemical nature of OMAA has been misunderstood that very little in the way of synthetic modification of the aldehyde group has been reported since the preparation of OMAA was first described by Wieland and Fahrmeir in 1970. The only report of a modifi-

Fig. II-4. Proton magnetic resonance spectrum of OMAA-II in d_6 -DMSO. TMS was used as the standard.



cation of this kind has been the synthesis of a dinitrophenylhydrazone in modest yield by reaction of a periodate oxidation product of AMA with dinitrophenylhydrazine (Morris and McSwine, 1983).

Until recently, OMAA has been thought to be a true aldehyde. Faulstich et al. (1973) proposed that the altered conformation of OMAA (as evidenced by its unique CD spectrum) was due to an intramolecular hydrogen bond between the carbonyl function of the aldehyde and the peptide backbone. Garritty and Brown (1978) reported an infrared spectrum of OMAA with bands at 2800 cm^{-1} and 1350 cm^{-1} which were thought to arise from the carbonyl of the aldehyde group. This view has been challenged by Morris and McSwine (1983) who were unable to detect a signal characteristic of an aldehydic hydrogen in the PMR spectrum of a periodate oxidation product of AMA. They have proposed that the aldehyde of aldoamanitins exists primarily as an intramolecular adduct with a nucleophile such as an amide nitrogen (e.g., Fig. II-5). The data presented in this chapter support this hypothesis, but do not provide direct proof of it.

An important indication that OMAA-I does not exist primarily as a free aldehyde is its stability. OMAA-I has been manipulated and stored for years in the ambient atmosphere in our laboratory without loss of the material. This is atypical of most true aldehydes, which usually must be stored under an inert atmosphere to prevent oxidation.

The available data suggest that the two forms of OMAA-I, although chromatographically distinct, are very similar in other respects. They both resemble OMAA, as it has been previously described by others, in terms of CD spectrum, color reaction with cinn-HCl, and reduction to form OMDA (Wieland and Fahrmeir, 1970; Faulstich et al., 1973). The

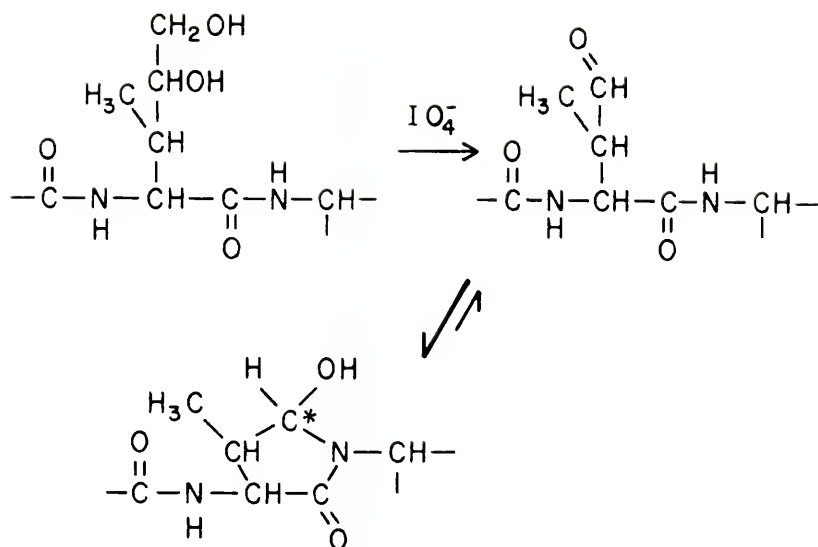


Fig. II-5. Scheme of periodate oxidation of the dihydroxyisoleucine sidechain and hypothetical intramolecular reaction of the aldehyde group. The asterisk marks the new chiral center formed by the intramolecular reaction.

studies with the HPLC-purified forms of OMAA-I have shown that they slowly interconvert in solution at room temperature. An equilibrium between the two components is reestablished from one of the purified forms over the course of several hours. This slow rate of interconversion is in sharp contrast with the short period of time (no more than a few minutes) which is required to establish the equilibrium following the periodate oxidation of OMA in neutral solution. A simple explanation for this discrepancy might be that neither form of OMAA-I is the entity first generated by the periodate oxidation (i.e., the free aldehyde), but rather they derive from it. Thus, the free aldehyde, when formed under conditions of near-neutral pH, rapidly and almost completely converts to the two forms of OMAA-I. The components of OMAA-I might be interconverting by a slow back-reaction to the free aldehyde followed by formation of the other component.

The PMR spectrum of OMAA-I in d_6 -DMSO showed only a very small signal with a chemical shift which would be expected for an aldehyde. Thus, our PMR spectral data confirm those of Morris and McSwine (1983). The inertness of OMAA-I to reaction with sodium chlorite has provided a further piece of evidence that supports the hypothesis that it is not primarily a free aldehyde.

The discovery that periodate oxidation of OMA under mildly acidic conditions generates a distinct entity has added greatly to our understanding of the nature of OMAA. This novel product, OMAA-II, appears to be a free aldehyde by spectroscopic (characteristic signal by PMR) and chemical (oxidation to carboxylic acid by sodium chlorite) criteria. The demonstration by both TLC and CD spectroscopy that OMAA-II changes to OMAA-I within minutes of neutralization of the solution

lends credence to the hypothesis outlined above that the periodate oxidation of OMA yields a free aldehyde (OMAA-II) which converts rapidly to the two forms of OMAA-I in solutions of near-neutral pH.

The finding that the CD spectrum of OMAA-II is indistinguishable from that of OMA is very interesting. This suggests that the presence of the aldehyde group per se does not cause the alteration in the toxin's conformation. This indicates that the hydrogen bond hypothesis for the altered conformation (Faulstich et al., 1973) is probably incorrect. The altered conformation of OMAA-I is associated with several characteristics, including a PMR spectrum which is not characteristic of an aldehyde, inertness to oxidation by sodium chlorite, and the presence of two similar, yet chromatographically distinguishable, forms. The probable explanation for this behavior of OMAA-I is that it exists in solution as a mixture of two diastereomers which are generated by the reaction of the aldehyde moiety with an intramolecular nucleophile. The α -amino group of the hydroxytryptophan residue is a reasonable candidate as this nucleophile on several grounds. As shown in Fig. II-5, reaction of the aldehyde group with this amide nitrogen to yield an alkanolamide would form a five-membered ring (thermodynamically favored?) with a new chiral center arising from the γ -carbon of residue 3 (marked with an asterisk). The generation of this additional chiral center provides for the possibility of two chromatographically separable diastereomeric forms. There have been reports of the ready formation of cyclic alkanolamides from the periodate oxidation of an appropriate diol (e.g., van Tamalen et al., 1960). This proposal for the structure of OMAA-I also provides a ready rationale for both the inertness of OMAA-I to sodium chlorite and the stability of

OMAA-II at mildly acidic pH's. The interconversion between alkanol-amides and their constituent aldehyde and amide are known to proceed very slowly at mildly acidic pH's, but at much higher rates with increasing pH (Hubert et al., 1975; Jencks, 1969, p. 495). Thus, at the mildly acidic pH's at which the oxidation with sodium chlorite was attempted OMAA-I was locked in the alkanolamide form and could not be oxidized. On the other hand, the reduction of OMAA-I with sodium borohydride was conducted at near-neutral pH where the conversion to free aldehyde would be permitted. When OMA is oxidized in an acidic medium, the formation of the alkanolamide is inhibited and OMAA-II, the free aldehyde, can be isolated or oxidized by sodium chlorite. However, when a solution of OMAA-II is neutralized the thermodynamically more stable OMAA-I rapidly forms.

Although the proposed structure of OMAA-I is strictly hypothetical, it is worthwhile to consider how the toxin's conformation might be altered by this sort of intramolecular reaction. First, crystallographic (Kostansek et al., 1978; Wieland et al., 1983) and NMR spectroscopic (Wieland et al., 1983) studies indicate that the α -amino of the hydroxytryptophan residue is hydrogen bonded to the terminal carbonyl of the asparagine residue. Formation of the alkanolamide would necessarily disrupt this hydrogen bond and perhaps destabilize the native conformation. Also, formation of the alkanolamide would generate what has been referred to as a "bridged lactam". Methods for intentionally introducing bridged lactams into peptides have been recently developed and applied to the synthesis of peptides with conformationally constrained backbones (Freidinger et al., 1980; Freidinger et al., 1982).

As will be seen in the next chapter, the chemical nature of OMAA-I seems to have a very large impact upon the rate and course of its reductive amination. Additional observations will be presented and discussed which indirectly support the proposed structure of OMAA-I.

CHAPTER III
TRANSFORMATIONS OF 6'-O-METHYLALDO- α -AMANITIN
VIA REDUCTIVE AMINATION AND OXIDATION

Introduction

Much of our knowledge about the relationship between structure and activity of amatoxins which differ at residue 3 (residue 3 corresponds to the dihydroxyisoleucine residue of AMA) has derived from the existence of three naturally-occurring variants in this sidechain (Fig. I-1, R₂). These variants correspond to three levels of hydroxylation: (1) no hydroxylation, (2) a hydroxyl group on the γ -carbon, or (3) hydroxyl groups on both the γ - and δ -carbon atoms (Wieland, 1983). Increasing extent of hydroxylation has been found to correlate with a modest increase in inhibitory activity toward CT RNAP II (Cochet-Meilhac and Chambon, 1974). In addition, the semisynthetic derivative OMDA has been prepared which contains a γ -hydroxyvaline residue. This hydroxyvaline residue is a close homolog of the isoleucine residue of amanullin. Thus, it is not surprising that OMDA and 6'-O-methylamanullin (OML) have similar inhibitory activity toward CT RNAP II (Cochet-Meilhac and Chambon, 1974). A totally synthetic amatoxin analog which has norvaline (an amino acid without a β -branch) at this position is a very poor inhibitor of RNAP (Wieland et al., 1981). However, the significance of this latter finding is unclear, since the effect of this structural change on the overall conformation of the peptide has not been examined. One can see that these data relate to

variations in the structure of this sidechain which are both very conservative and which result in small changes in inhibitory activity. In particular, no charged groups had been introduced into this residue at the time that this work was begun.

Although the exact nature of the site on the RNAP molecule to which amatoxins bind has not been elucidated, some information about the manner in which amatoxins inhibit RNAP has been gathered. Although their mode of inhibition is apparently noncompetitive (Cochet-Meilhac and Chambon, 1974), the amatoxins may inhibit RNAP by binding to a catalytic subsite. Both genetic (Greenleaf, 1983) and biochemical (Brodner and Wieland, 1976) studies have implicated one of the large subunits, which presumably contributes part of the active site, as being involved in amanitin binding. Also, some recent work has led to the proposal that amatoxins may inhibit RNAP by binding to a portion of the catalytic site which is involved in translocation of the nascent RNA molecule (Vaisius and Wieland, 1982).

Since the amatoxins bear no close resemblance to RNAP's substrates or products, it has been difficult to conceptualize their mechanism of inhibition and to generate a hypothetical framework upon which studies of structure-activity relationships can be planned. Thus, although reductive coupling of OMAA to protein amino groups was envisioned as a possibly useful route for the conjugation of amatoxins to proteins, there did not exist a large body of knowledge concerning the likely effect of this kind of modification on the activity of the toxin nor was there a theoretical basis upon which to predict such effects. In addition, the results presented in the previous chapter suggested that the forms of OMAA which could be useful for conjugation

to protein amino groups, e.g., OMAA-IA and OMAA-IB, do not have a free aldehyde function and also that the form of OMAA which has a free aldehyde, OMAA-II, is present in solutions of neutral pH in very small quantities. It was reasonable to anticipate that this diminished availability of the aldehyde group would have a negative impact upon the rate of reductive coupling to amines. However, it was difficult to predict a priori the magnitude of this effect.

In order to assess the importance of these factors, the reductive coupling of OMAA-I to some simple amines was examined (Fig. III-1). In addition, two oxidative transformations of the aldehyde group (to carboxyl and nitrile functions) have been accomplished. These two additional alterations of this sidechain provide conservative changes in the structure and additional important information about the structural requirements for potent inhibition of RNAP II.

Material and Methods

Materials

Reagents. Ammonium acetate, glycine, and L-proline were obtained from Sigma Chemical. Sodium cyanoborohydride (Sigma Chemical) was recrystallized (Jentoft and Dearborn, 1979) and kept desiccated over P_2O_5 . Hydroxylamine-O-sulfonic acid was purchased from Alfa Chemical. Triethylamine (TEA) was obtained from Pierce Chemical. The source of other reagents has been described in the previous chapter.

Toxin. The syntheses of OMA and OMAA have been described in the previous chapter. These two compounds served as the starting material for all of the semisynthetic derivatives described here.

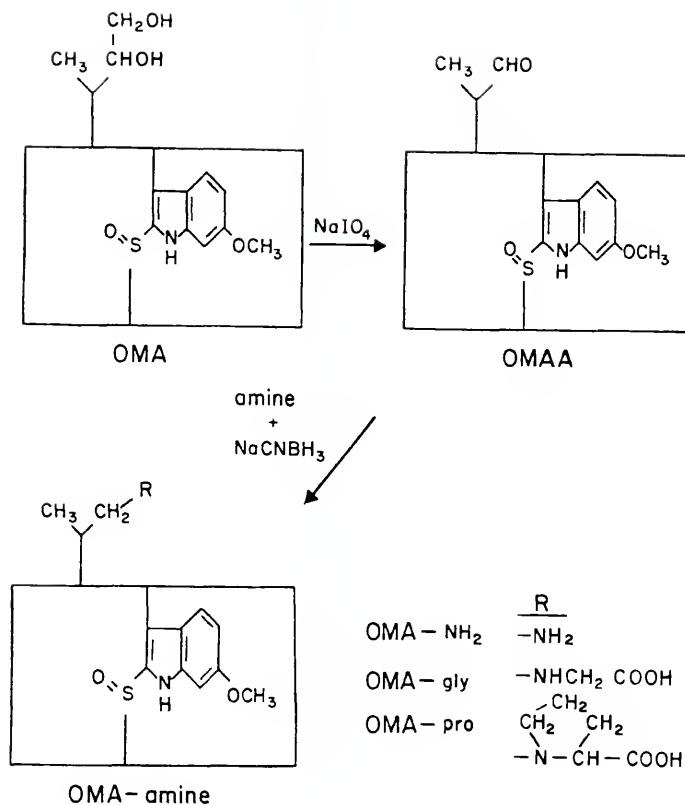


Fig. III-1. Scheme showing the reductive amination of OMAA.

Analytical Procedures

Spectroscopy and chromatography. Absorbance and CD spectroscopy and chromatographic procedures were performed as described in chapter II. TLC solvent system II contained 2-butanol: methanol: 0.5 M sodium chloride (4:2:1). Fast-atom bombardment mass spectroscopy (FAB-MS) was performed by Triangle Laboratories (Durham, NC) using a VG 7070E mass spectrometer. In some cases HPLC solvents contained a buffer instead of water; this buffer contained 20 mM TFA which was adjusted to pH 3.0 with TEA and will be referred to as TFA-TEA buffer (Cohen et al., 1983).

RNA polymerase assay. The analysis of inhibition of CT RNAP II was conducted as described in the previous chapter.

Synthesis of Amatoxin Derivatives

Desalting of toxin solutions. Certain toxin solutions were desalted (Böhlen et al., 1980) with the aid of Sep Pak C18 cartridges (Waters Associates). The C18 cartridges were prerinsed with 20 ml of 50% acetonitrile followed by 10 ml of water. Aqueous solutions of toxin were loaded onto the cartridge at a rate no greater than 1 ml/min. After the cartridge was washed with 15 ml of water, the toxin was finally eluted with 10 ml of 50% acetonitrile. When OMA-NH₂ was desalted, this protocol was modified in that 20 mM ammonium bicarbonate was substituted for water at each step.

Synthesis of OMA-NH₂. To 10 ml of molar methanolic ammonium acetate was added 6.3 mg (0.1 mmol) of sodium cyanoborohydride. A portion of this solution (1.0 ml) was added to 2.0 µmol of lyophilized OMAA. The reaction was allowed to proceed in the dark at 23°C for 18

days. The solution was concentrated to dryness at 30°C in vacuo, redissolved in acetonitrile: TFA-TEA buffer (22:78), and chromatographed by HPLC using the same solvent at 0.5 ml/min. The desired product eluted as the major product between 21 and 26 ml. This material was reconcentrated to dryness at 30°C in vacuo, dissolved in acetonitrile: TFA-TEA buffer (15:85), and rechromatographed by HPLC in this solvent at 0.5 ml/min. The toxin derivative eluted from the column between 45 and 57 ml. This was concentrated at 30°C in vacuo to remove the acetonitrile and then desalted using the appropriate Sep Pak C18 protocol (see above). The eluate from the Sep Pak C18 cartridge was concentrated at 30°C in vacuo to remove the acetonitrile, supplemented with 10 ml of water, and lyophilized. The final yield was 0.55 μ mol (28%). TLC: R_F , I-0.60, II-0.83; color-reaction, violet. FAB-MS: $[M+H]^+$ - expected, 902.37; found, 902.33; $[M-H]^-$ - expected, 900.37; found, 900.14.

Synthesis of OMA-gly. Glycine (0.751 g, 10 mmol) and sodium cyanoborohydride (31.5 mg, 0.5 mmol) were dissolved in sufficient 10% methanol to make 5.0 ml of solution. Lyophilized OMAA (2.0 μ mol) was dissolved in 0.10 ml of this solution. After 96 h in the dark at 23°C the reaction mixture was taken up in 22% acetonitrile and chromatographed by HPLC using the same solvent at 0.5 ml/min. The desired product eluted between 16 and 25 ml yielding 1.68 μ mol (84%). TLC: R_F , I-0.46, II-0.46; color-reaction, violet.

Synthesis of OMA-pro. A methanolic solution (0.10 ml) containing 0.5 M L-proline and 0.05 M sodium cyanoborohydride was added to 2.0 μ mol of OMAA with dissolution of the toxin. After 170 h in the dark at 23°C the reaction mixture was taken up in 22% acetonitrile and applied

to HPLC using the same solvent at 0.5 ml/min. The desired product, which eluted between 26 and 29 ml, was concentrated to dryness in vacuo at 30°C, redissolved in 15% acetonitrile, and rechromatographed by HPLC using this solvent at 0.5 ml/min. The product (1.45 μ mol, 73%) eluted between 69 and 77 ml. TLC: R_F , I-0.37, II-0.36; color-reaction, violet.

Synthesis of OMA-COOH. A solution (0.4 ml) containing 0.015 M sodium chlorite, 0.030 M amidosulfonic acid, and 0.10 M sodium phosphate, pH 2.0, was added to 2.0 μ mol of OMA. To this was immediately added 10 μ l of a solution containing 0.24 M sodium periodate and 0.1 M sodium phosphate, pH 2.0. After 10 min in the dark at 23°C the toxin solution was desalted using the Sep Pak C18 technique, concentrated to dryness in vacuo at 40°C, redissolved in acetonitrile: TFA-TEA buffer (18:82), and chromatographed by HPLC using the same solvent at 1.0 ml/min. The desired product eluted between 47 and 51 ml. After concentration in vacuo at 30°C to remove acetonitrile the toxin solution was desalted again to yield 0.85 μ mol (47%). TLC: R_F , I-0.49, II-:0.29; color-reaction, violet. FAB-MS: $[M+H]$ - expected, 917.34; found, 917.27; $[M-H]$ - expected, 915.33; found, 915.49.

Synthesis of OMA-CN. Hydroxylamine-O-sulfonic acid (22.6 mg, 0.2 mmol) was dissolved in sufficient 1.0 M sodium borate, pH 9.0, to make 1.0 ml of solution. A portion of this (0.20 ml) was used to dissolve 2.0 μ mol of lyophilized OMAA. After 72 h in the dark at 23°C the reaction mixture was taken up in 22% acetonitrile and chromatographed by HPLC using the same solvent at 1.0 ml/min. The desired product (1.16 μ mol, 58%) eluted between 36 and 45 ml. TLC: R_F , I-0.63, II-0.85; color-reaction, violet.

Results and Discussion

Reductive Amination of OMAA-I

Reaction of OMAA-I with ammonium acetate in the presence of sodium cyanoborohydride was the first reductive amination reaction attempted. This reaction would be expected to yield the primary amine OMA-NH₂ (Fig. III-2, structure VI with R=R'=H), the simplest amine accessible via this route. Several unexpected difficulties were encountered in the synthesis of OMA-NH₂. Even when ammonium acetate was included at a stoichiometry of 100 to 1 relative to OMAA, the major products appeared to be dimeric and/or trimeric products (cf. Borch et al., 1971). That is, they were bound by a cation-exchange resin (Sephadex SP-50), yet were unreactive with fluorescamine. Their appearance was suppressed by increasing the molar ratio of ammonium acetate relative to OMAA from 100 to 500. However, instead of a single fluorescamine-reactive entity being produced under these new conditions, there were several. The concern was that OMAA and/or the desired product were undergoing a secondary degradative reaction. Since it has been well documented that many peptides which contain residues of 2,4-diaminobutanoic acid are unstable at neutral pH (Davies et al., 1969; Katrukha et al., 1968; Poduska et al., 1965), it seemed plausible that OMA-NH₂, which contains the related 2,4-diamino-3-methylbutanoic acid residue, might display comparable lability. Another area of concern was the tryptathionine residue which is believed to be susceptible to base-catalyzed destruction (Wieland, 1983). However, when some of these byproducts were isolated by chromatography on Sephadex LH-20, many of them were found to be unstable and

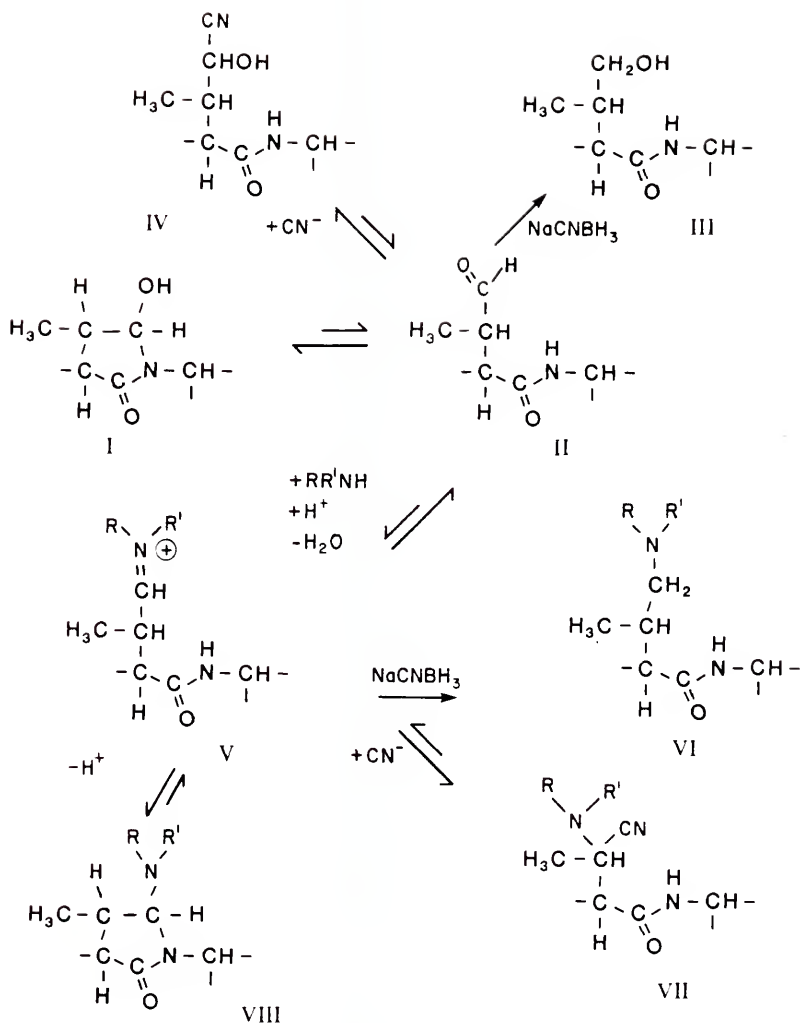


Fig. III-2. Scheme showing the probable reactions and side-reactions occurring in the reductive amination of OMAA.

to decompose back to the starting material, OMAA (data not shown). These quasi-stable amines may be α -aminonitriles (Fig. III-2, structure VII) (Bejaud et al., 1976) which have already been implicated as undesirable, quasi-stable byproducts in the reductive methylation of proteins (Gidley and Sanders, 1982). One or more of these amines might also arise from reaction between the intermediate immonium (Fig. III-2, structure V) and an intramolecular nucleophile (such as an amide nitrogen) to yield an N-Mannich compound (Tramontini, 1973). This intramolecular reaction to form an N-Mannich (Fig. III-2, structure VIII) is comparable to that proposed in chapter II for the formation of an intramolecular alkanolamide (Fig. III-2, structure I) in OMAA. Studies on the stability of N-Mannich compounds of this kind have been recently reported (Bundgaard, 1985; Loudon et al., 1981). Their stability increased with decreasing bulk of the substituents on the amino group (i.e., R and R' in Fig. III-2, structure VIII). The N-Mannich which might form during the synthesis of OMA-NH₂ would bear the smallest substituents possible (hydrogen) and thus impede the progress of the reaction on account of its high stability. When the reaction was permitted to progress for several weeks, most of these fluorescamine-reactive compounds slowly disappeared and one increased in amount. As noted above, chromatographic separation on Sephadex LH-20 of the components of a reaction which still contained multiple fluorescamine-reactive amatoxin derivatives showed that many of these entities were unstable and decomposed back to the starting material, OMAA. This supports the notion that labile compounds like structures VII and VIII (Fig. III-2) are formed. Probably as a consequence of these or similar side-reactions, the purified yield of OMA-NH₂ has

never exceeded 30% despite numerous efforts to optimize the reaction.

Reductive coupling of OMAA to the amino acids glycine and L-proline proceeded at a much higher rate than the reaction with ammonium acetate. This higher rate may derive, in part, from the larger substituents on amino group and a consequent lowering of the stability of the N-Mannich byproduct. When these reactions were fractionated by HPLC before the reaction reached completion, novel compounds which decomposed to OMAA were again noted and in this case isolated from void-volume fractions. OMA-gly and OMA-pro can now be obtained in approximately 75% yield.

Oxidative Transformations of OMAA

Reaction of OMAA-I with hydroxylamine-O-sulfonic acid produced OMA-CN in 60% yield after a period of a few days. This reaction relies upon the formation of an O-sulfonated oxime by reaction of the reagent with the aldehyde. This then undergoes an elimination reaction to yield the nitrile and a sulfate ion (Fizet and Streith, 1974).

OMAA-II was generated by the reaction of OMA with sodium periodate at pH 2.0 (see previous chapter). By inclusion of sodium chlorite (Laurer and Tomimatsu, 1956; Lindgren and Nilsson, 1973) in this system, OMAA-II was oxidized quickly to the carboxyl derivative, OMA-COOH.

Properties of the Derivatives

These derivatives had chromatographic properties expected for their structures. The charged derivatives generally migrated more slowly on TLC and had shorter retention times on HPLC than OMAA, while

the relatively nonpolar OMA-CN showed the opposite behavior. In contrast to OMAA-I, the CD spectra of all of the new derivatives were indistinguishable from that of OMA (Fig. II-3). Also, their color-reaction with cinn-HCl was violet compared with the rust color obtained with OMAA-I. As expected, only OMAA-NH₂ reacted with fluorescamine to yield a fluorescent product. FAB-MS of OMA-COOH and OMA-NH₂ revealed mass ions of the appropriate size for the proposed structures (Figs. III-3 and III-4).

Instability of the reductive amination products has been detected after prolonged storage of frozen solutions. This destruction was associated with loss of the distinctive UV absorbances, e.g., at 304 nm (data not shown). Loss of the compounds was retarded when solutions were mildly acidic.

None of the new derivatives inhibited CT RNAP II as strongly as AMA. OMA-CN was the strongest inhibitor with a K_i of 3×10^{-9} M. OMA-gly, OMA-pro, OMA-COOH, and OMA-NH₂ were much weaker inhibitors with K_i 's of 2.5×10^{-7} M, 7×10^{-6} M, 1×10^{-7} M, and 1.7×10^{-7} M, respectively. The inhibition by OMA-gly, OMA-pro, and OMA-NH₂ was studied more thoroughly and showed apparently noncompetitive behavior (e.g., Fig. III-5). Since the amanitin amines were considerably less potent inhibitors than other amanitins which might be generated in small amounts as byproducts of the reaction (e.g., OMDA), steps were taken to help ensure that a K_i obtained for the material in a peak was characteristic of the bulk of the material in the peak and not grossly biased by the presence of traces of more potent inhibitors. In order to accomplish this, the K_i was determined for the contents of at least two, and usually three, fractions across a peak. The material in the

Fig. III-3. Fast atom bombardment mass spectra of OMA-COOH in the positive ion (panel A) and negative ion (panel B) modes.

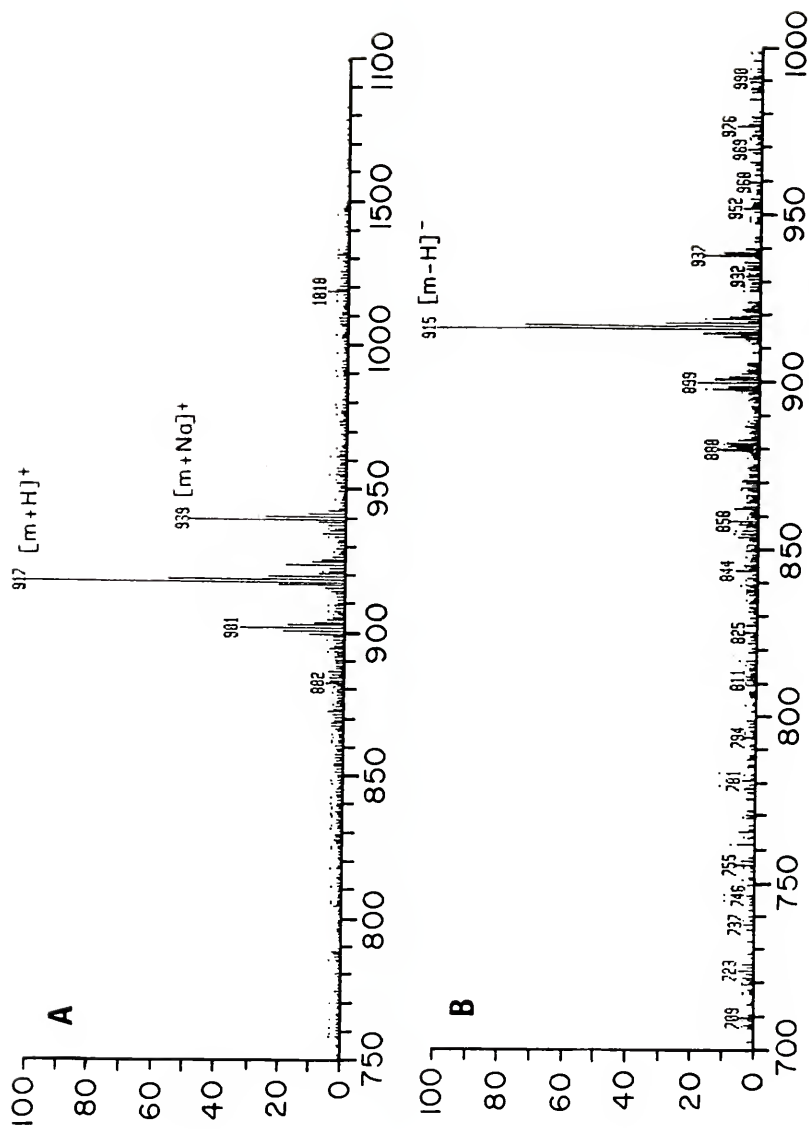
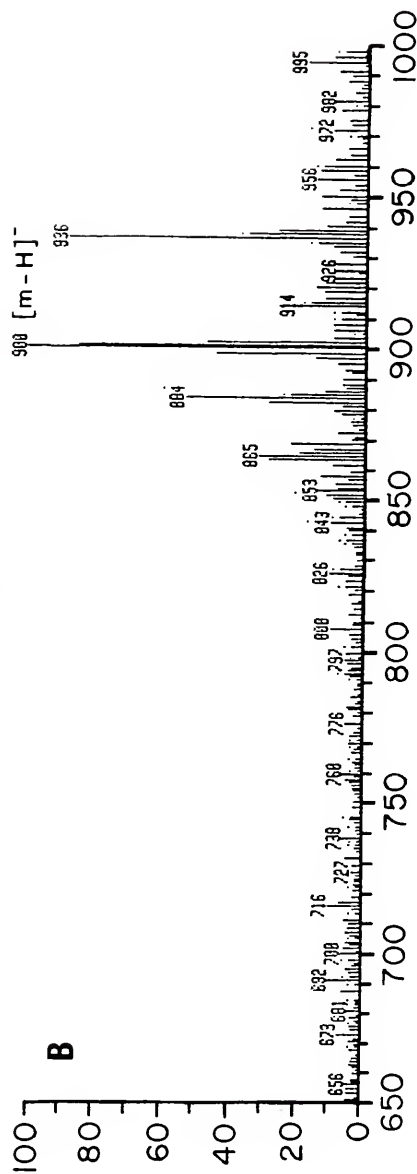
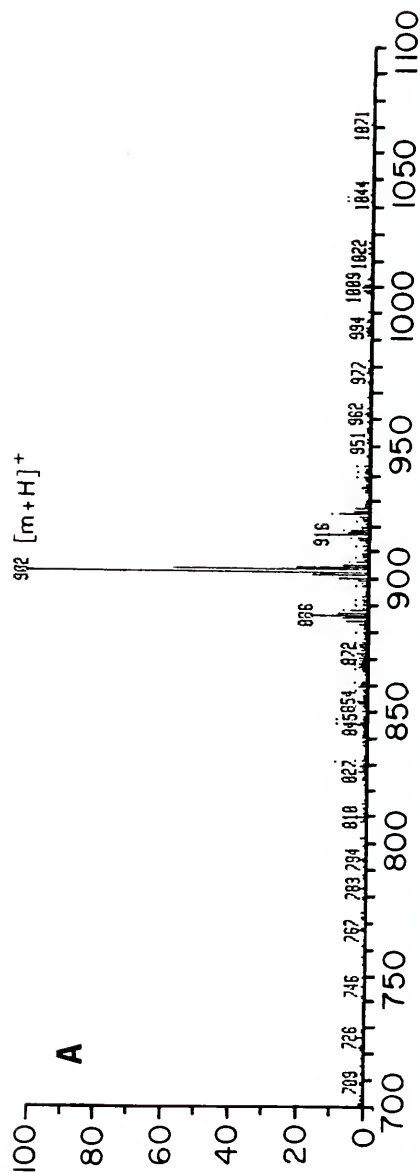


Fig. III-4. Fast atom bombardment mass spectra of OMA-NH₂ in the positive ion (panel A) and negative ion (panel B) modes.



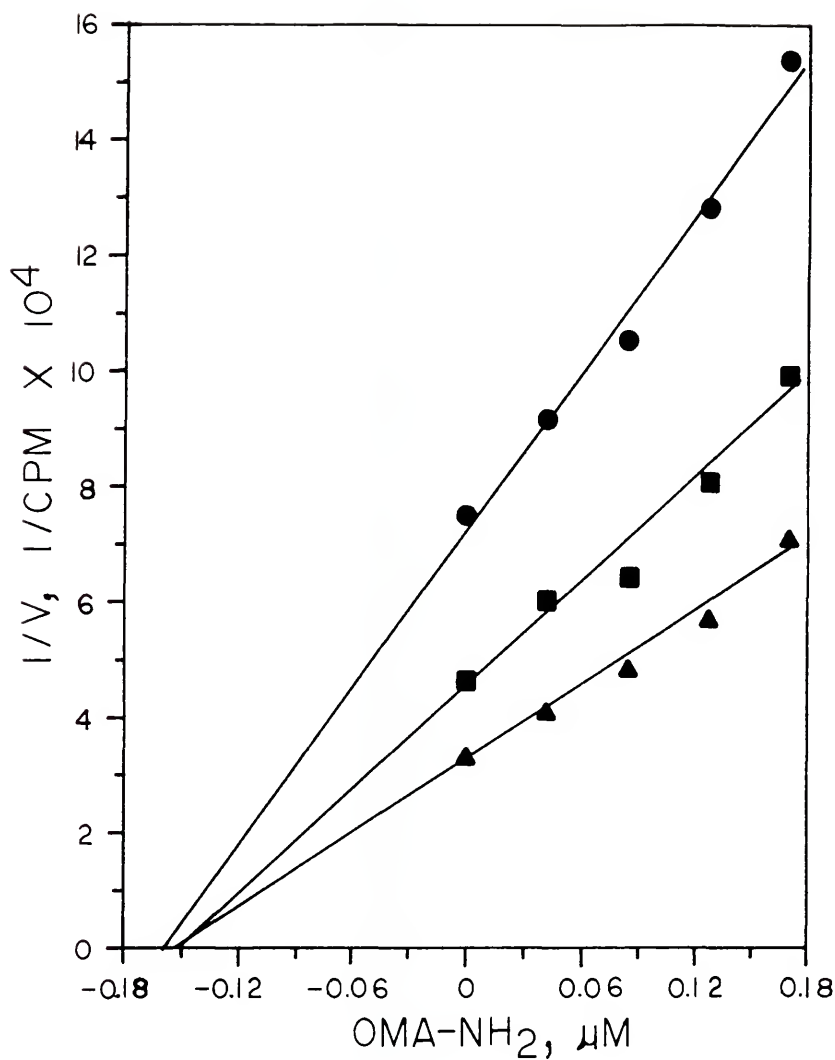


Fig. III-5. Dixon plot showing the apparently noncompetitive inhibition of CT RNAP II by OMA-NH₂. The concentrations of UTP used were 0.75 μM (circles), 1.10 μM (squares), and 1.50 μM (triangles).

peak was subjected to additional chromatographic steps until it appeared homogeneous with respect to inhibition of CT RNAP II. The K_i of the pooled fractions from each final peak was consistent with that of the constituent fractions (data not shown).

The finding that the inhibition of CT RNAP II by each of the amanitin amines showed noncompetitive behavior with respect to UTP provides preliminary evidence that these amanitin derivatives inhibit this enzyme in a manner analogous to other amanitins, presumably through localization at an amanitin binding site on the enzyme. This evidence for inhibition by a mechanism common to other amanitins, along with the resumption of a "native" conformation as suggested by the CD spectra, provides a basis for interpreting their diminished affinities in terms of interactions between the enzyme and the newly introduced substituents. In this regard, OMA-NH₂ is most readily compared to the previously examined amanitins, OMDA and OML. These three derivatives differ only with respect to a single group. They each have a different group of roughly the same size and shape appended to the γ -carbon atom of residue 3 (see Figs. I-1 and III-1). While amanitins OMDA and OML, which have a hydroxyl and a methyl group, respectively, at this location, bind to CT RNAP II almost as tightly as the most inhibitory of the naturally-occurring toxins, OMA-NH₂ which bears an amino group is a much poorer inhibitor. At first glance, the primary difference between OMA-NH₂ and the other two amanitins appears to be the positive charge expected for an aliphatic amino group at near-neutral pH. However, because of its charge the amino group may also be effectively somewhat larger than the analogous hydroxyl and methyl groups due to a larger layer of bound water. Thus,

it is difficult to attribute the difference in affinity unequivocally to the presence of a positive charge, but this seems likely to be the most important factor. The K_i 's of OMA-gly and OMA-pro are much more difficult to rationalize because of the multiplicity of functional groups which have been introduced and because of the high likelihood of chelation of the Mn^{2+} in the RNAP assay system by the amino acid moieties.

Since OMA-NH₂, which bears a positive charge, was a relatively poor inhibitor of CT RNAP II, it was anticipated that OMA-COOH, which should bear a negative charge, would be a quite potent inhibitor. It was surprising to find that it inhibits the enzyme almost as poorly as OMA-NH₂. In contrast, OMA-CN, a semisynthetic derivative which is like OMDA in that it lacks a group with a charge, is a rather potent inhibitor of CT RNAP II. These data taken together suggest that the portion of the amanitin binding site of CT RNAP II which interacts with the sidechain of residue 3 may contain closely spaced positively and negatively charged moieties. Thus, derivatives with charged sidechains are strongly repulsed, while derivatives with uncharged sidechains can bind more strongly. Alternatively, this portion of the binding site may be somewhat nonpolar so that the addition of highly polar substituents to this sidechain inhibits binding.

It seems clear that OMAA is not well-suited for reductive coupling to proteins. Even under forcing conditions with very high concentrations of amines, the rate of reaction is very slow. However, some the derivatives described here and future subderivatives may prove useful in a number of different applications.

CHAPTER IV

STUDIES ON THE CONJUGATION OF N-ACYLATED AMINO SUGARS TO BOVINE SERUM ALBUMIN BY MEANS OF REDUCTIVE ALKYLATION

Introduction

The results presented in the previous chapter indicated that reductive coupling of OMAA to protein amino groups does not represent a feasible route for the conjugation of amanitin to proteins. Consequently, attention was turned to the preparation of an azo amanitin derivative which could be employed in this way.

Synthesis of azo amanitins requires diazotization of an aromatic amine. However, since aldehydes are reactive to diazotizing reagents, the aldehyde group needed to be introduced in protected or precursor form. This need for protection is complicated by the fact that amatoxins are unstable toward most of the conditions which are used to deprotect aldehydes or to generate them from a precursor (Wieland, 1983).

Many aldoses contain aldehyde groups which are inherently protected by intramolecular reaction with a hydroxyl group to form a hemiacetal. Despite the fact that only a very small proportion of an aldohexose (e.g., glucose) is present in solution in the free aldehyde form, these sugars have been successfully coupled to proteins by reductive alkylation with sodium cyanoborohydride (Gray, 1974). Under the conditions which were initially described, this reaction was quite slow. However, Roy et al. (1984b) have recently noted that borate

increases the amount of free aldehyde which is in equilibrium with the cyclic forms of aldoses. This effect was most prominent for glucose and lactose. They were further able to show that borate greatly increased the rate of reductive coupling of lactose to BSA (Roy et al., 1984a).

These observations by Roy and his coworkers suggested a possible route for preparing an azo amanitin with the desired properties. A derivative of the already well-studied ABGG was envisioned which would bear an amino sugar linked to the carboxyl group of the glycylglycine linker (see Fig. V-I). This chapter describes experiments which were undertaken to define the feasibility and optimal reaction conditions for the reductive coupling of N-acylated amino sugars to protein. Model compounds which contain the N-4-nitrobenzoylglycylglycine moiety linked to an amino sugar (D-galactosamine or D-glucosamine) were prepared and utilized for these studies. The conjugation of these compounds to protein amino groups is schematically depicted in Fig. IV-1. The effects of temperature, pH, buffer, and various reactant concentrations on the reaction rate have been studied.

Materials and Methods

Reagents

N-4-nitrobenzoylglycylglycine (PNBGG) was obtained from United States Biochemicals. Nickel (II) chloride, BSA, sodium cyanoborohydride, 2-amino-2-deoxy-D-glucose (glucosamine) hydrochloride, 2-amino-2-deoxy-D-galactose (galactosamine) hydrochloride, 1-ethyl-3-(3-dimethylamino)propylcarbodiimide (EDC), N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), and sodium 3-trimethyl-

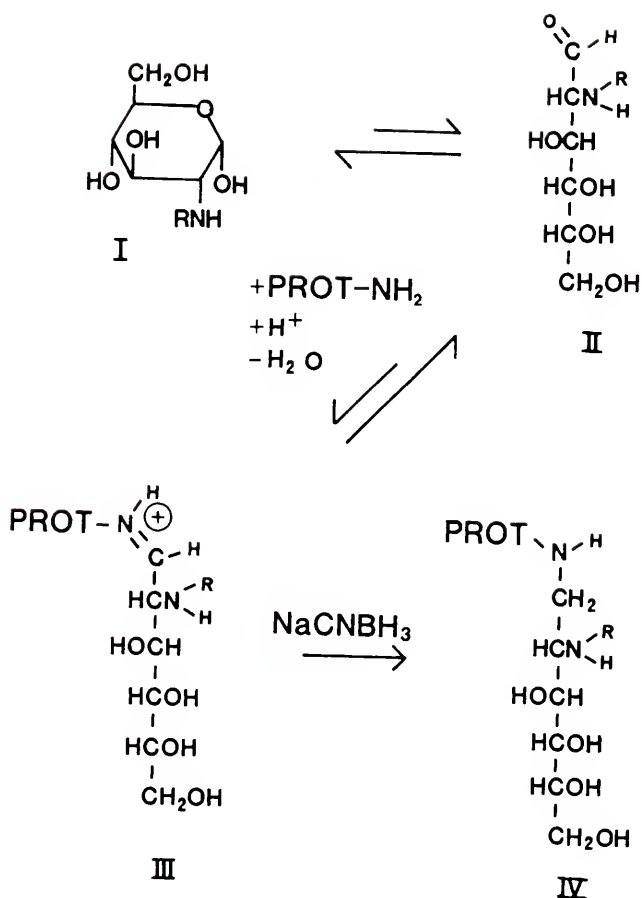


Fig. IV-1. Scheme illustrating the reductive coupling of PNBGG-sugar derivatives (or ABGG-GLU) to proteins; "R" represents the PNBGG (or ABGG) moiety.

silyl-d₄-propanoate (TSP) were obtained from Sigma Chemical. Sodium cyanoborohydride was recrystallized according to Jentoft and Dearborn (1979). Trichloroacetic acid (TCA) was obtained from Fisher Scientific. Other reagents were obtained as described in the previous chapters.

Analytical Procedures

Chromatography. TLC and HPLC were performed as described in the previous chapters. TLC solvent system III contained 1-butanol: methanol: water (4:2:1); solvent system IV contained 2-butanol: ethyl acetate: water (14:12:5). Column chromatography was also performed with Sephadex LH-20 and Sephadex SP-50 (Pharmacia Fine Chemicals). Bio-Beads SM-4 (Bio-Rad) were soxhlet-extracted with methanol for seven days prior to use.

Spectroscopy. Spectroscopic studies were performed as outlined in the previous chapters. In addition, optical rotation was determined with a Jasco DIP-360 digital polarimeter equipped with a 10 cm cell.

Elemental analysis. Elemental analysis was performed by the instrument facility in the Department of Chemistry, University of Florida.

Synthesis of PNBGG Derivatives

Synthesis of PNBGG-GLU. To PNBGG (0.56g, 2.0 mmol) in 4.0 ml of water was added with stirring 0.18 ml of 10 M NaOH, EDC (0.575 g, 3.0 mmol), and glucosamine HCl (0.86 g, 4.0 mmol). After 12 h of reaction at 23°C in the dark the resultant gel was dissolved in 100 ml of water. The solution was added to 125 ml of Bio-Beads SM-4, stirred

for 1 h, and then filtered. The resin was washed for another hour with 100 ml of water and filtered again. Bound material was eluted from the resin by extracting six times for 10 min with 100 ml of 75% methanol. The combined methanolic extracts were concentrated in vacuo at 40°C to a small volume. After filtration through Whatman # 40 paper the solution was applied to a 2.5 X 15 cm column of Sephadex SP-50 (Na^+) and eluted with 100 ml of water. The eluant was concentrated in vacuo at 50°C to a small volume and applied to a 2.5 X 95 cm column of Sephadex LH-20 which was eluted with water. Fractions of 7.5 ml were collected at approximately 0.2 ml/min. The desired product (1.35 mmol, 67%) was located in fractions 52 to 60. TLC: R_F , III-0.68, IV-0.34. For $\text{C}_{17}\text{H}_{22}\text{N}_4\text{O}_{10}$ calculated: C 46.16, H 5.01, N 12.66; found: C 45.22, H 5.13, N 12.32. $[\alpha]^{25} = +21^\circ$. $\epsilon_{268} = 1.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. PMR (Fig. IV-2): 3.46-3.99 (m, 6H, sugar); 4.04 (s, 2H, glycine); 4.21 (s, 2H, glycine); 4.77 (d, 0.4H, $J=8.2 \text{ Hz}$, anomeric); 5.20 (d, 0.6H, $J=3.3 \text{ Hz}$, anomeric); 8.04 (d, 2H, $J=8.6 \text{ Hz}$, aromatic); 8.37 (d, 2H, $J=8.6 \text{ Hz}$, aromatic).

Synthesis of PNBGG-GAL. The synthesis of PNBGG-GAL was similar to that of PNBGG-GLU. PNBGG-GAL eluted from the Sephadex LH-20 column in fractions 59 to 65 (57% yield). TLC: R_F , III-0.68, IV-0.34. For $\text{C}_{17}\text{H}_{22}\text{N}_4\text{O}_{10}$ calculated: C 46.16, H 5.01, N, 12.66; found: C 45.10, H 5.11, N 12.23. $[\alpha]^{25} = +32^\circ$. $\epsilon_{268} = 1.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. PMR (Fig. IV-3): 3.6-4.3 (m, 10H, sugar and glycines); 4.70 (d, 0.5H, $J=8.4 \text{ Hz}$, anomeric); 5.24 (d, 0.5H, $J=3.6 \text{ Hz}$, anomeric); 8.03 (d, 2H, $J=8.6 \text{ Hz}$, aromatic); 8.36 (d, 2H, $J=8.9 \text{ Hz}$, aromatic).

Fig. IV-2. Proton magnetic resonance spectrum of PNBGG-GLU in D_2O .

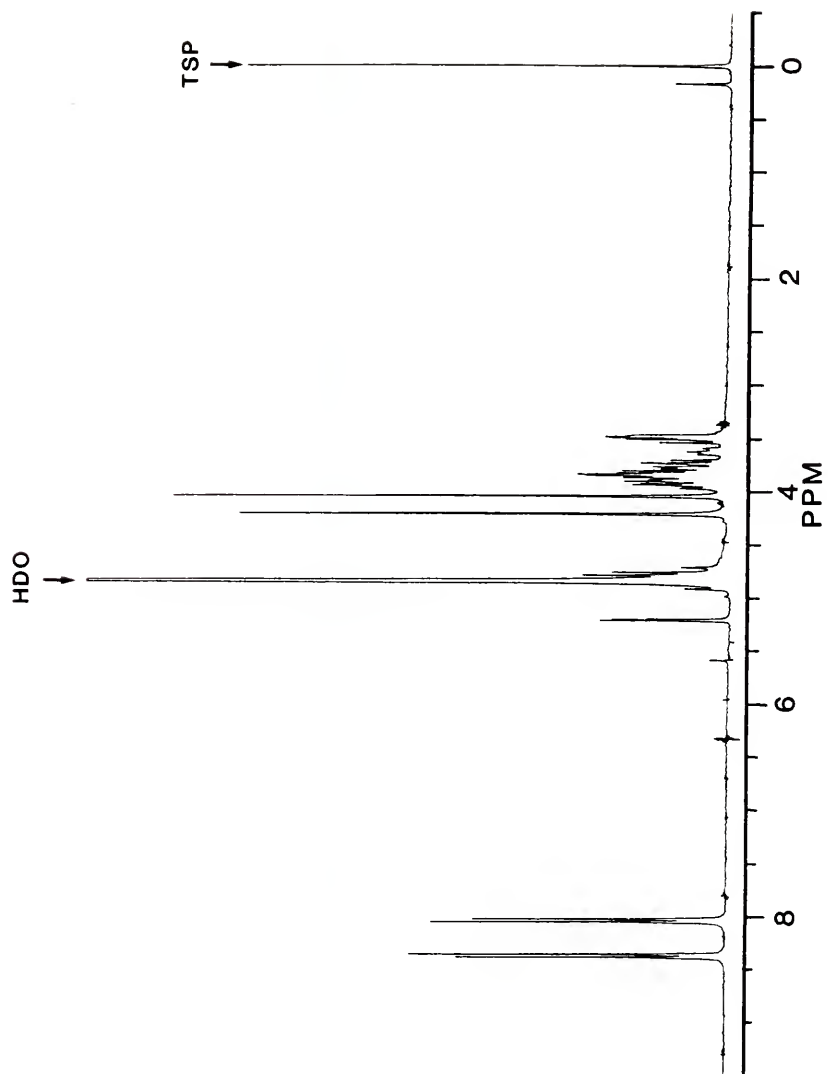
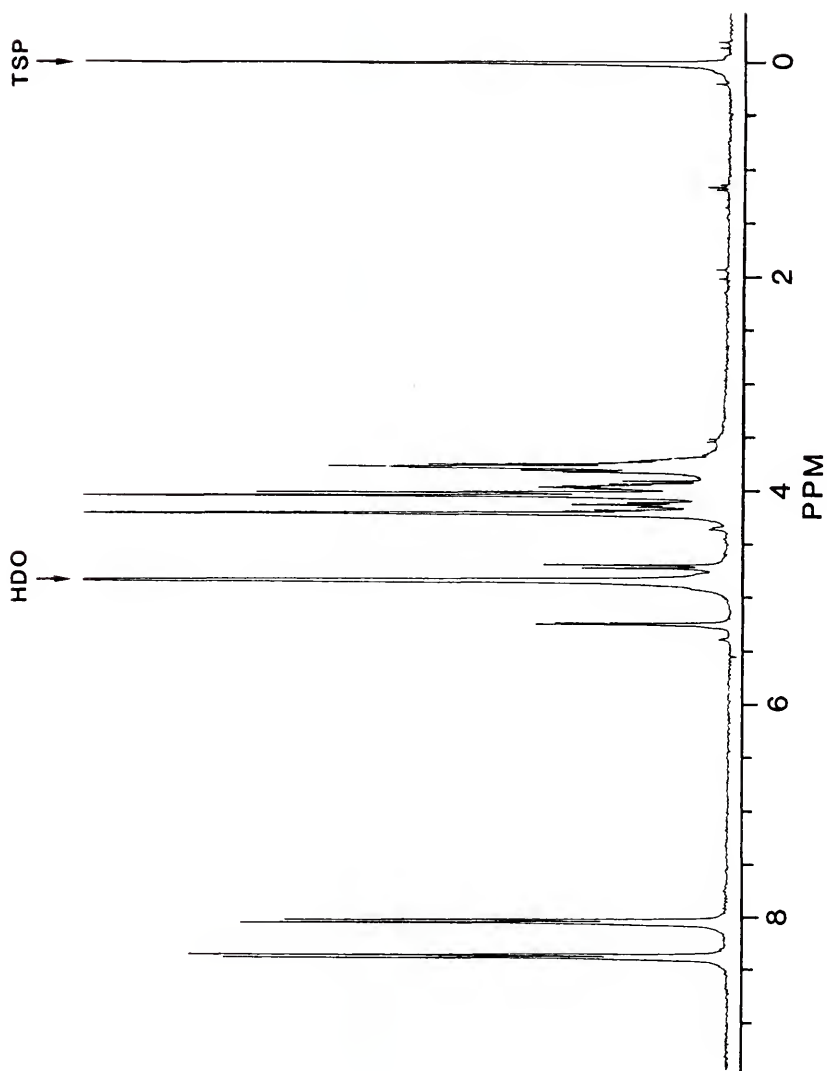


Fig. IV-3. Proton magnetic resonance spectrum of PNBGG-GAL in D_2O .



Conjugation Protocols

Conjugation of PNBGG-GLU and PNBGG-GAL to BSA. BSA stocks were dialyzed against water or the appropriate buffer before use. Molar concentrations of BSA were estimated on the basis of $E^{1\%}$ of 6.67 at 279 nm (Janatova et al., 1968) and a molecular weight of 66,300 (Reed et al., 1980). Reaction components were sterilized by filtration through 0.22 μ m Millex-GV filters (Millipore) and added aseptically to autoclaved vials. All reactions contained 1.0 mg/ml (1.51×10^{-5} M) BSA and 3.62×10^{-3} M of a carbonyl compound (PNBGG-GLU or PNBGG-GAL). Other components were incorporated as indicated below. Reactions were allowed to proceed in the dark at either 20°C or 37°C.

Purification and analysis of conjugates. The BSA conjugates were freed of unconjugated PNBGG derivatives by one of two methods. In experiments where the buffer concentration was 0.2 M, samples of 200 μ l were removed aseptically and placed on ice. The chilled solution was adjusted to 10% TCA with 40 μ l of ice-cold 60% TCA. After 15 min the precipitate was pelleted by centrifugation for 5 min in a table-top centrifuge (Fisher Scientific). The supernatant fluid was decanted and the pellet dissolved in 0.1 M sodium phosphate, pH 7.0. This precipitation with TCA was repeated and the final pellet was dissolved in 0.5 ml of the sodium phosphate buffer.

In experiments where buffer concentrations exceeded 0.2 M the conjugate was purified with Centricon CM-30 ultrafiltration devices (Amicon). The sample (0.2 ml) of the reaction mixture was added to the device and diluted to 2.0 ml with 0.2 M sodium borate, pH 8.0, with mixing. This was then centrifuged at 5,500 rpm for 2 h in either a Beckman JA-40 or Sorvall type 30 rotor at 5°C in order to concentrate

the conjugate solution to approximately 50 μ l. The conjugates were diluted and concentrated three more times in a similar fashion. The second wash was with 0.1 M disodium ethylenediaminetetraacetic acid (EDTA), while the third and fourth washes were with 0.1 M sodium phosphate, pH 7.0. The final concentrate was diluted with 0.5 ml of 0.1 M sodium phosphate, pH 7.0

The extent of coupling was estimated by comparing the absorbances at 279 nm and 300 nm. Molar extinction coefficients for BSA at 279 nm and 300 nm were determined to be approximately $4.54 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $3.0 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. The comparable values for the PNBGG derivatives were found to be $1.0 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and $3.95 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. These extinction coefficients reflect a significant difference in the absorbance spectra of BSA and the PNBGG derivatives. The absorbance of the PNBGG derivatives falls slowly in the near UV (Fig. IV-4), while that of BSA falls rapidly. It is this difference which has been exploited to provide a method for estimating the extent of conjugation. Simultaneous equations which incorporated these extinction coefficients were solved to yield an algorithm (cf. Mishell and Shiigi, 1980, pp. 345-7) by which the absorbance at 300 nm could be partitioned into the contributions from BSA and the conjugated PNBGG derivative: $A_{300} \text{ from PNBGG} = 1.21(A_{300}) - 0.0832(A_{279})$. Based upon the contribution to A_{300} from each component and their respective extinction coefficients, the molar ratio of PNBGG derivative to BSA in the conjugate was easily calculated. Absorbance measurements at 279 nm and 300 nm were corrected for light scattering by extrapolation from 390 nm, a wavelength at which neither BSA nor the PNBGG derivatives absorb significantly. Light scattering was assumed to obey Rayleigh's

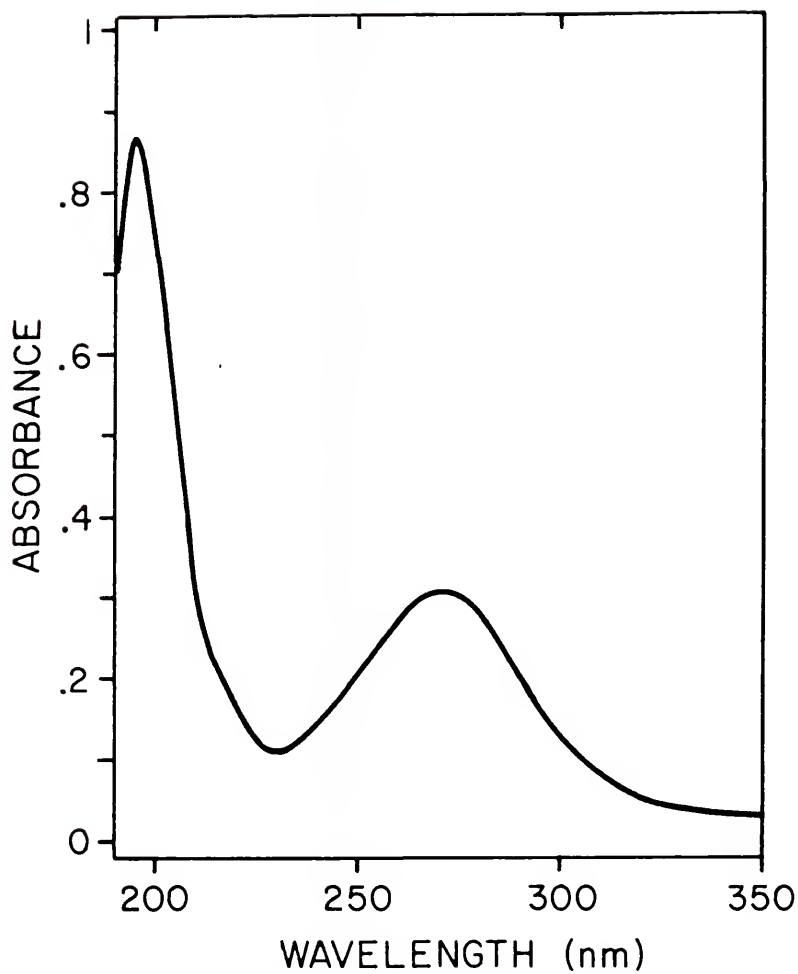


Fig. IV-4. UV absorbance spectrum of PNBGG-GLU in water at approximately 2.5×10^{-5} M.

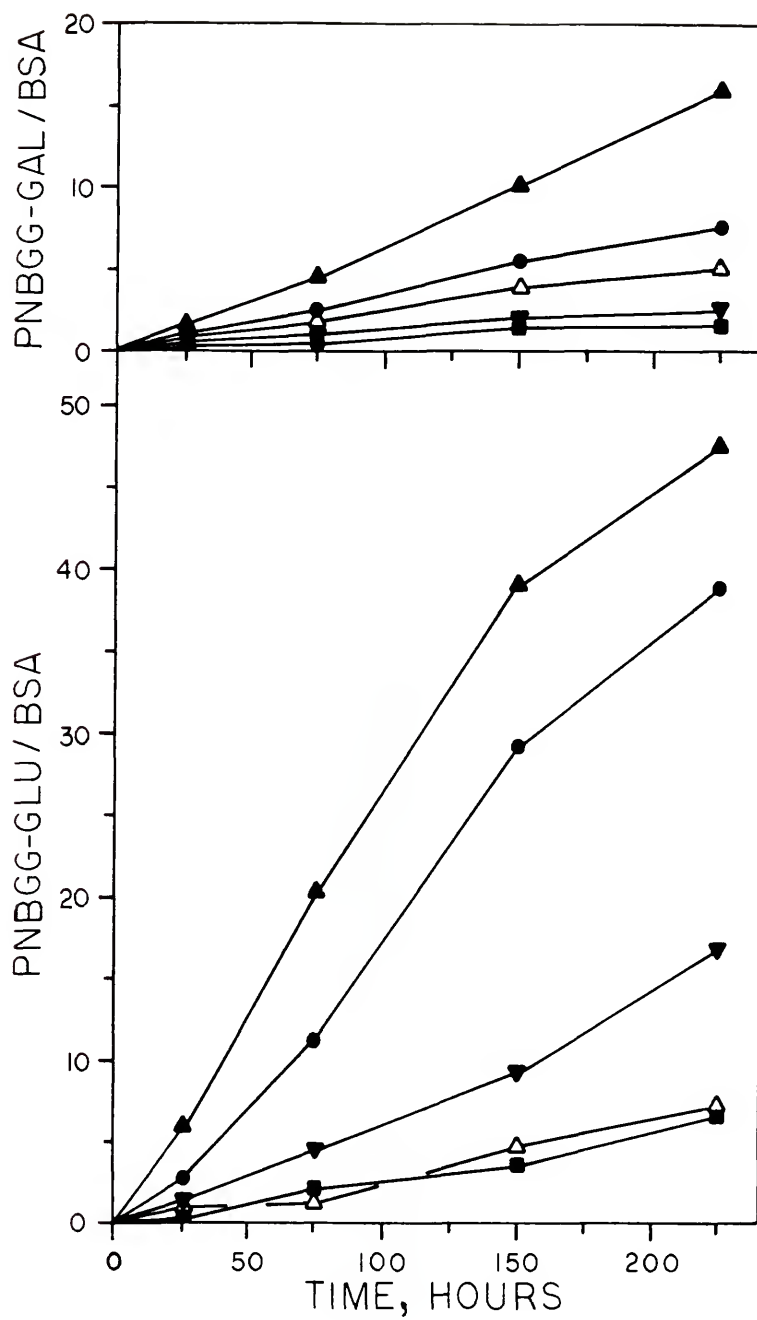
theorem ideally and thus to vary with the fourth power of wavelength (cf. Leach and Scheraga, 1960).

Results

Initial Studies on the Effects of pH, Temperature, Nature of Sugar Residue, Borate, and Sodium Cyanoborohydride Concentration on the Conjugation of PNBGG-Sugars to BSA

The results of initial studies to examine the influence of several reaction parameters that were thought likely to be important are summarized in Fig. IV-5. Conjugation of PNBGG-GLU and PNBGG-GAL to BSA was found to be strongly dependent upon sodium cyanoborohydride. In the absence of sodium cyanoborohydride there was a slow time-dependent increase in the extent of conjugation, but the degree of conjugation after 225 hours was less than three PNBGG-sugar molecules per BSA molecule for all of the conditions examined. There was no significant change in the A_{279} or A_{300} (i.e., the spectral parameters used to follow the course of the reaction) of the BSA in sham reactions which lacked PNBGG-GLU or PNBGG-GAL or in reactions where lactose was conjugated to the BSA (data not shown). The most important factors affecting the rate of conjugation were the use of borate instead of other buffer salts (phosphate or HEPES) and the temperature (reactions proceeded much more rapidly at 37°C than at 20°C). In accord with the results of Roy et al. (1984b), the effect of borate on the reaction rate was more marked for the glucosamine than for the galactosamine derivative. In fact, in the presence of borate PNBGG-GLU coupled at a significantly higher rate than PNBGG-GAL, while there was little difference in their rates of reaction in the absence of borate. The influence of pH (pH 8.0 versus pH 9.0) was found to be less signifi-

Fig. IV-5. Effect of pH, temperature, and nature of sugar residue on the extent of conjugation of PNBGG-GAL (upper panel) and PNBGG-GLU (lower panel) to BSA over time. All reaction mixtures contained 1.0 mg/ml BSA, 3.62 mM PNBGG derivative, 7.24 mM sodium cyanoborohydride, 10 mM nickel (II) chloride, and 6 mM sodium citrate in a 0.2 M buffer. Closed symbols represent borate buffer-containing reactions, while the open upright triangle contained phosphate buffer. Upright triangle, pH 9.0 at 37°C; circle, pH 8.0 at 37°C; inverted triangle, pH 9.0 at 20°C; square, pH 8.0 at 20°C.



cant, but reactions did proceed more rapidly at pH 9.0. The inclusion of nickel (II) chloride as a cyanide-absorbing reaction component (Jentoft and Dearborn, 1980) had no demonstrable negative effect upon either the reaction rate or the properties of the conjugate (data not shown).

Further Studies on the Effect of Sodium Cyanoborohydride
Concentration, Borate Concentration, and pH on the Conjugation of
PNBGG-GLU to BSA

The initial studies described above showed that the glucosamine residue permitted much higher reaction rates than the galactosamine residue. Thus, further work was restricted to PNBGG-GLU. The dependence of rate on the sodium cyanoborohydride concentration is presented in Fig. IV-6. Under the conditions utilized the rate increased rapidly with increasing sodium cyanoborohydride concentration and leveled off at approximately 0.10 M. Furthermore, under conditions of optimized sodium cyanoborohydride concentration there was no significant effect of varying borate concentration between 0.2 M and 2.0 M on the rate of coupling (Fig. IV-7). Variation of the pH between 6.0 and 9.0 had a profound effect on the rate of conjugation (Fig. IV-8). While there was a large increase in rate between pH 7.0 and 9.0, at pH's less than 7.0 the rate was very low, almost negligible.

Discussion

The coupling of sugars to proteins by reductive alkylation with sodium cyanoborohydride was first demonstrated by Gray (1974). Even with concentrations of the reactants at near saturation the rate of

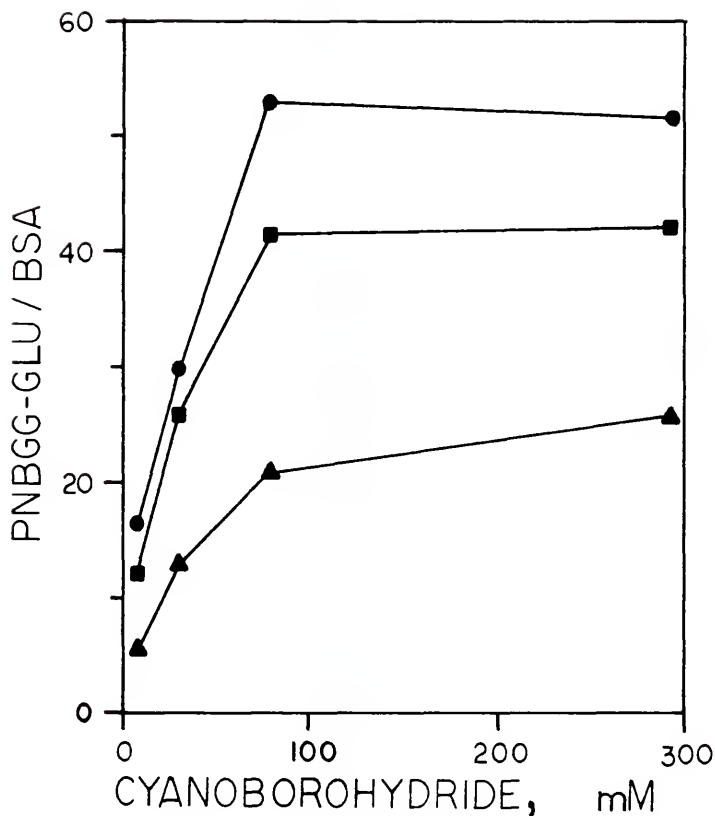


Fig. IV-6. Effect of sodium cyanoborohydride concentration on the extent of conjugation of PNBGG-GLU to BSA after various times. All reactions were conducted with 1 mg/ml BSA, 10 mM nickel (II) chloride, and 6 mM sodium citrate in 0.2 M sodium borate, pH 8.0, at 37°C. The triangles, squares, and circles represent samples at 25, 50, and 75 hours of reaction, respectively.

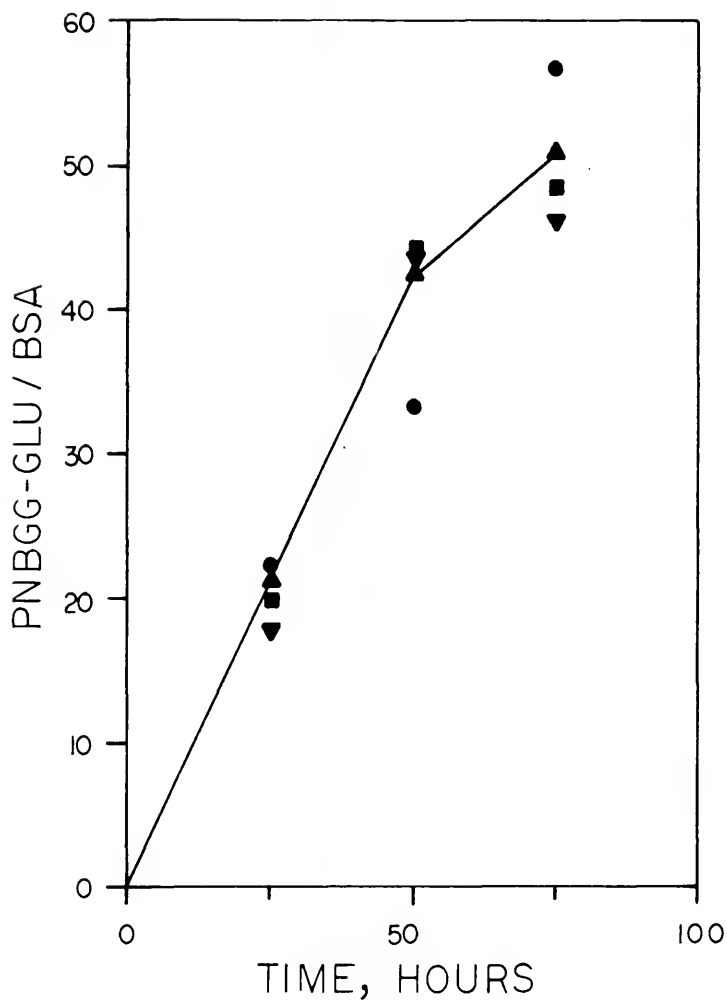


Fig. IV-7. Effect of borate concentration on the extent of conjugation of PNBGG-GLU to BSA at various times. All reactions were conducted with 1 mg/ml BSA, 10 mM nickel (II) chloride, 6 mM sodium citrate, and 72.5 mM sodium cyanoborohydride in borate buffers of varying concentration with pH 8.0 and temperature 37°C. Inverted triangle, 0.2 M; square, 0.5 M; upright triangle, 1.0 M; and circle, 2.0 M sodium borate.

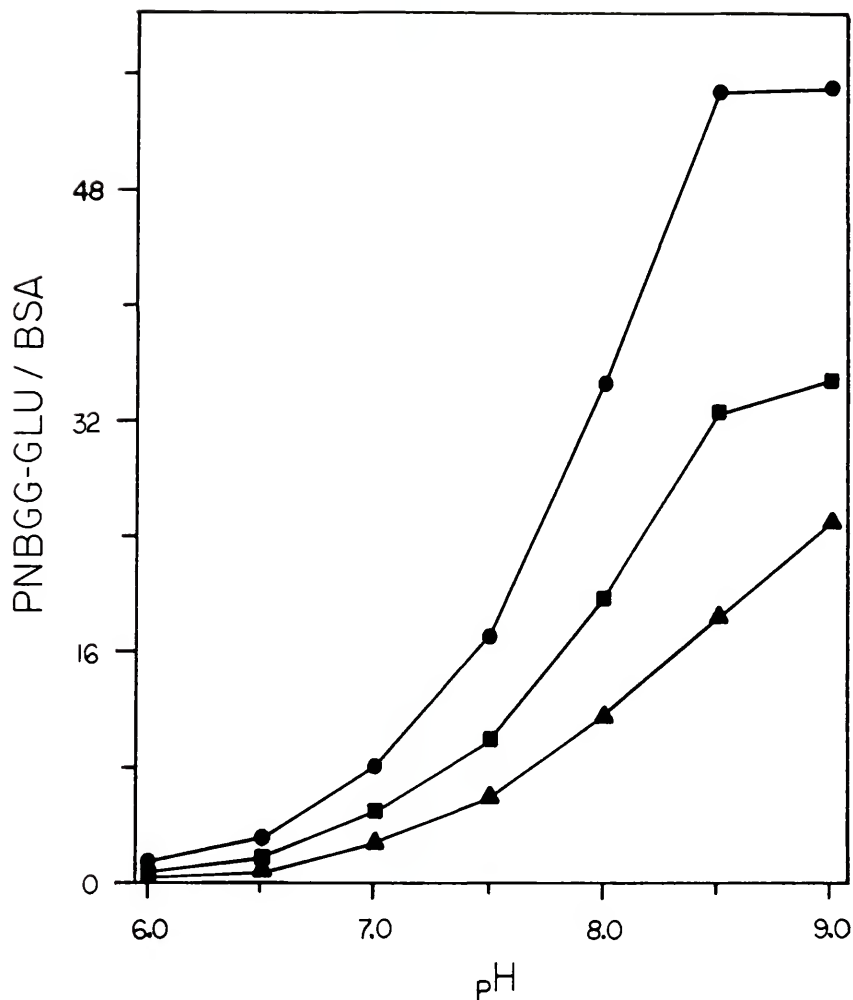


Fig. IV-8. Effect of pH on the extent of conjugation of PNBGG-GLU to BSA at various times. All reaction mixtures contained 1 mg/ml BSA, 0.1 M sodium cyanoborohydride, 10 mM nickel (II) chloride, and 6 mM sodium citrate in a buffer consisting of 0.2 M sodium borate and 0.05 M PIPES. All reactions were kept at 37°C at the appropriate pH. The triangles, squares, and circles represent samples analyzed after 25, 50, and 75 hours of reaction time, respectively.

conjugation was very slow. Despite this sluggishness, the linkage of sugars to proteins by this method has served as an important route for the synthesis of neoglycoproteins. The demonstration by Roy and his coworkers (1984a, 1984b) that borate increases the proportion of acyclic sugar in solution and thus greatly accelerates the rate of reductive coupling of lactose to BSA has served to enhance the attractiveness of this approach for conjugation of sugars to proteins. If this phenomenon of borate-enhanced reductive coupling might also apply to N-acylated 2-amino-2-deoxy-D-aldohehexoses, then its utility could conceivably be extended to the conjugation of a wide variety of compounds to proteins.

The PNBGG adducts of glucosamine and galactosamine were prepared as synthetic intermediates of the corresponding ABGG derivatives. These proved to have characteristics which made them useful for preliminary studies on the coupling of this class of compounds to protein. In particular, they were quite soluble in water and their UV absorbance spectra were sufficiently different from that of BSA to permit estimation of the extent of conjugation based upon spectral parameters.

The reductive coupling of both PNBGG-GLU and PNBGG-GAL was markedly stimulated by 0.2 M borate. As might be expected from the studies of Roy et al. (1984b), PNBGG-GLU reacted substantially more rapidly than PNBGG-GAL in the presence of borate, while their rate of reaction was similar in solutions of other buffers. Increasing the temperature from 20°C to 37°C was also found to greatly enhance the rate of coupling, while increasing the pH from 8.0 to 9.0 had a much smaller effect. The reductive coupling of the PNBGG derivatives to BSA

was strongly cyanoborohydride-dependent, but very slow, time-dependent conjugation of the PNBGG derivatives to BSA was detected in the absence of sodium cyanoborohydride. This reaction may correspond to that previously seen in solutions of glucose with BSA (Baynes et al., 1984).

Optimization of the conjugation reaction was examined further with the more active PNBGG-GLU. The reaction rate was seen to increase markedly as the sodium cyanoborohydride concentration was increased to approximately 0.1 M, beyond which the rate plateaued. This finding is in sharp contrast to reductive methylation with formaldehyde and sodium cyanoborohydride where the rate varies little with the cyanoborohydride concentration (Jentoft and Dearborn, 1979). However, there have been indications of a similar strong dependence on cyanoborohydride concentration for the rate of reductive coupling of raffinalldehyde to proteins (van Zile et al., 1979). Under conditions of optimized sodium cyanoborohydride concentration at pH 8.0, increasing the borate concentration from 0.2 to 2.0 M had no significant effect upon the rate.

A more extensive examination of the effect of pH was undertaken. This showed a large increase in the rate of coupling between pH 7.0 and 8.0. Previous studies on the reductive coupling of sugars to proteins in buffers other than borate have noted a significant increase in the rate with increased pH (Marsh et al., 1977; Schwartz and Gray, 1977). This presumably resulted from the fact that sugars have a higher proportion of their acyclic form in solution at higher pH's (Roy et al., 1984b). The pH-dependence of conjugation noted here may derive in part from this effect, but it is likely to be much more

strongly related to the pH-dependence of the interaction between borate and the sugar.

This study, in contrast with others, has focused upon the reductive coupling of complex compounds to proteins using relatively low reactant concentrations. Most previous workers have utilized concentrations which could only be achieved with sugars and highly soluble proteins. The current studies have defined a system in which compounds can be coupled to proteins at a practical rate using reactant concentrations which may be readily achieved with drugs (or their derivatives) and immunoglobulins. The optimized rate of conjugation attained here with PNBGG-GLU and BSA appears to approach the rate obtained by Lee and Lee (1980) for reductive coupling of some free aldehydes to BSA.

From the work of Hall (1956) on the pKa of various amines it can be anticipated that the pKa of protein amino groups which have been linked to one of the PNBGG derivatives will drop to about 9.0. This should provide considerable preservation of charge at physiologic pH.

In the next chapter the application of this method to the conjugation of amanitin to BSA is described.

CHAPTER V

CONJUGATION OF A NOVEL AZO AMANITIN TO BOVINE SERUM ALBUMIN VIA REDUCTIVE ALKYLATION WITH SODIUM CYANOBOROHYDRIDE

Introduction

The results obtained with the model compounds in the preceding chapter suggested that preparation of a derivative of AMA containing D-glucosamine would provide a reasonable route to an amatoxin which could be conjugated to proteins by means of reductive alkylation. Preparation of the amino analog of PNBGG-GLU (PABGG-GLU) has permitted access to a simple synthetic route to an azo amanitin derivative, ABGG-GLU, which bears the D-glucosamine residue. This new compound is closely related chemically to the azo amanitin ABGG which has already been successfully utilized to prepare several conjugates that have demonstrated potent and specific cytotoxicity (see chapter I).

The conjugation of ABGG-GLU to BSA was studied in order to permit direct comparisons with the data obtained with the model compounds. The behavior of ABGG-GLU in this system appeared more complex than that of PNBGG-GLU in some important respects. Therefore, further studies were undertaken to identify and optimize parameters which were felt likely to be particularly relevant to the conjugation of ABGG-GLU.

Materials and Methods

Reagents

N-4-aminobenzoylglycylglycine (PABGG) was obtained from Dr. James F. Preston and was prepared by hydrogenation of PNBGG as previously described (Preston et al., 1981). Piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) was obtained from Sigma Chemical. Other reagents were obtained or prepared as indicated in previous chapters.

Analytical Procedures

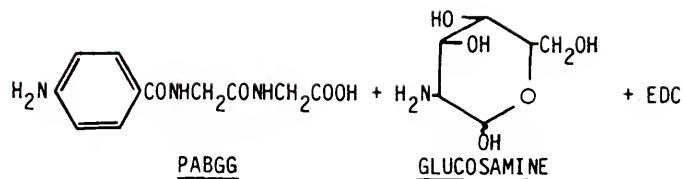
Chromatography, spectroscopy, and elemental analysis. These methods are fully described in previous chapters.

RNA polymerase assay and source of toxin. The protocols for examining the inhibition of RNA polymerase activity and for purification of AMA are outlined in chapter II.

Synthesis of ABGG-GLU

Synthesis of PABGG-GLU. Two methods for the synthesis of PABGG-GLU have been developed (Fig. V-1):

In the first method PABGG (0.126 g, 0.50 mmol) and glucosamine hydrochloride (10.8 g, 50 mmol) were added to 43.5 ml of water in a flask. The PABGG was brought into solution by dropwise addition of 0.55 ml of 10 M NaOH with continuous rapid stirring. EDC (0.192 g, 1.0 mmol) was added to this solution four times at 30 min intervals; during this time the reaction was allowed to proceed at 23°C in the dark. After a total reaction time of 2 h the solution was concentrated in vacuo at 30°C to near dryness. Remaining water was removed as an azeotrope with ethanol by twice suspending the residue in 25 ml of



OR

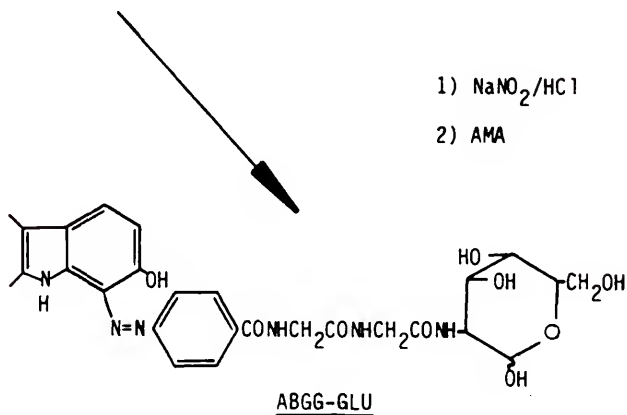
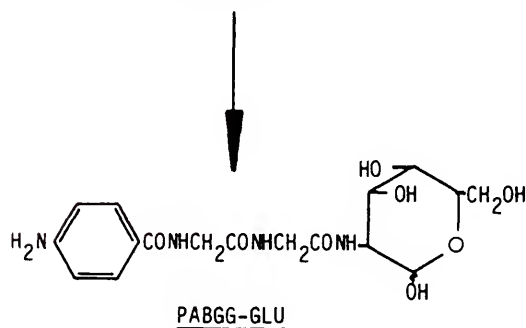
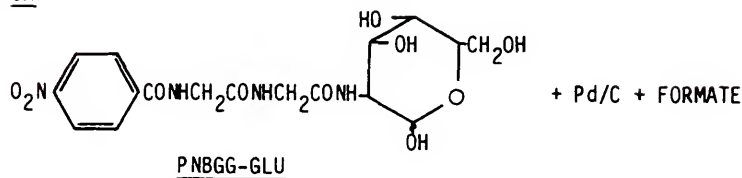


Fig. V-1. Scheme showing the two routes for synthesis of PABGG-GLU and the synthesis of ABGG-GLU from AMA and PABGG-GLU.

absolute ethanol and evaporating in vacuo at 30°C. The dried residue was suspended in 50 ml of methanol and filtered through Whatman # 40 paper. The filter cake was washed twice with 25 ml of methanol at room temperature. The pooled filtrates were concentrated to dryness in vacuo at 30°C, dissolved in 10 ml of water, and applied to a 4 X 20 cm column of Sephadex SP-50 (Na^+) which was eluted with 500 ml of water. This aqueous Sephadex SP-50 eluate was concentrated in vacuo at 40°C to a small volume, applied to a 2.5 X 95 cm column of Sephadex LH-20, and eluted with water at 0.2 ml/min to collect 7.5 ml fractions. The desired product (0.19 mmol, 38%) eluted between fractions 53 and 60.

A second method of preparing PABGG-GLU involved catalytic transfer hydrogenation (cf. Anwer and Spatola, 1980) of PNBGG-GLU. To PNBGG-GLU (0.50 mmol) in 45 ml of water was added 5 ml of 1.0 M sodium formate, pH 3.5. The solution was sparged with nitrogen for 15 min and then 0.375 g of 10% Pd on charcoal (Kodak) was added. This mixture was stirred for 12 h in the dark at 23°C under a nitrogen atmosphere. The catalyst was then removed by filtration through Whatman # 40 paper; the catalyst was washed with 50% acetonitrile until the A_{278} of the filtrate was negligible. The combined filtrates were concentrated to a small volume in vacuo at 50°C and applied to a 2.5 X 95 cm column of Sephadex LH-20. Fractions of 7.5 ml were collected at approximately 0.2 ml/min. The desired product (0.41 mmol, 82%) eluted in fractions 51 to 58. TLC: R_F , I-0.31, III-0.56; . For $\text{C}_{17}\text{H}_{24}\text{N}_4\text{O}_8$ calculated: C 49.51, H 5.87, N 13.58; found: C 47.23, H 6.28, N 13.18. $[\alpha]^{25}_{\text{D}} = +16^\circ$. $\epsilon_{278} = 1.38 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. PMR: 3.4-4.0 (m, 6H, sugar); 4.015 (s, 2H, glycine); 4.12 (s, 2H, glycine); 4.75 (d, 0.4H, $J=8.0$ Hz, anomeric);

5.20 (d, 0.6H, $J=3.3$ Hz; anomeric); 6.87 (d, 2H, $J=8.4$ Hz, aromatic); 7.70 (d, 2H, $J=8.6$ Hz, aromatic).

Synthesis of ABGG-GLU. PABGG-GLU (30 μmol) was dissolved in 0.75 ml of ice-cold 0.1 M HCl. The PABGG-GLU was diazotized by addition of 30 μl of 1 M sodium nitrite followed by incubation at 23°C in the dark for 30 min. A portion of this solution (0.65 ml) was added to 25 μmol of AMA which was dissolved in 1.20 ml of 0.5 M sodium phosphate, pH 8.0. There was immediate development of a deep purple color. After 5 min the crude product was desalted using the Sep Pak C18 protocol (see chapter III). The desalted material was dried in vacuo at 50°C, redissolved in acetonitrile: TFA-TEA buffer (18:82), and purified by HPLC. The desired product (13.8 μmol , 55%) eluted between 32 and 40 ml. After evaporation of the acetonitrile in vacuo at 30°C the final product was desalted by the Sep Pak C18 procedure. TLC: I-0.17, III-0.44. FAB-MS: $[\text{M}+\text{H}]^+$ - expected, 1342.50; found, 1342.54; $[\text{M}-\text{H}]^-$ - expected, 1340.485; found, 1340.50.

Conjugation of ABGG-GLU to BSA

Reaction mixtures were assembled as described in the previous chapter except that ABGG-GLU was substituted for the PNBGG derivatives. Excess ABGG-GLU was removed from the conjugates by the ultrafiltration method which was detailed in chapter IV. The only modification made in this protocol was that the conjugates were incubated in the presence of EDTA for a full hour before the second centrifugation.

The extent of conjugation was estimated spectrophotometrically. The molar extinction coefficient of ABGG-GLU at 395 nm was taken to be $1.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ (Faulstich and Trischmann, 1973); the extinction at

279 nm in 0.1 M sodium phosphate, pH 7.0, was determined to be $1.23 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. The $E^{1\%}$ at 279 nm and molecular weight of BSA (see chapter IV) were used to calculate a molar extinction at 279 nm of $4.54 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$; BSA does not absorb at 395 nm. Using these parameters the molar ratio of ABGG-GLU to BSA in the conjugates was estimated by the formula: $3.24A_{395}/(A_{279}-0.88A_{395})$.

The fate of ABGG-GLU in the reaction mixtures was determined by analytical HPLC. Duplicate samples (2-10 μl each) were diluted in 0.1 M sodium phosphate, pH 3.0, to yield a final concentration of approximately 0.2 mM ABGG-GLU. The diluted samples were applied to HPLC by an automated sample injector (Waters Model 710B WISP) and chromatographed at 1 ml/min on a C18 Z-module (Waters) using acetonitrile: TFA-TEA buffer (18:82) as the eluant. The absorbance at 304 nm was monitored and peaks were integrated by a Waters Model 720 Data Processor.

Results

Synthesis and Properties of ABGG-GLU

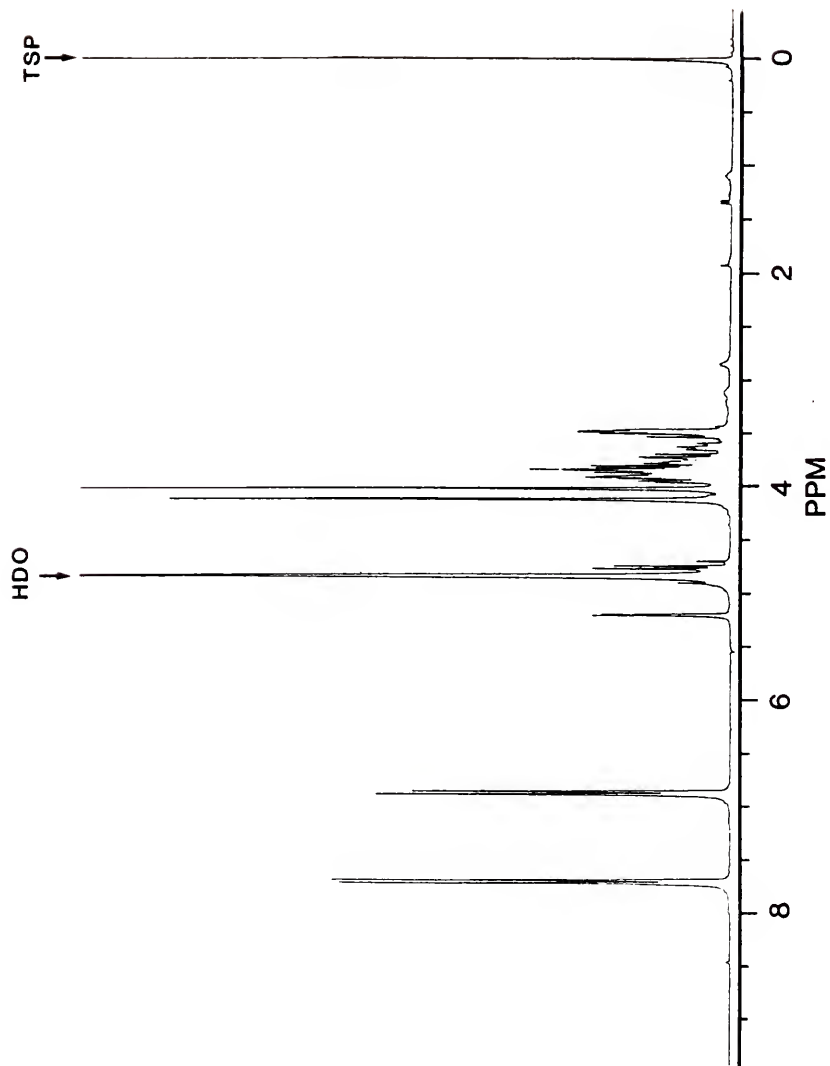
Preparation of PABGG-GLU. Two routes for the synthesis of PABGG-GLU have been developed (Fig. V-1). When initial efforts to reduce PNBGG-GLU failed, a first method was developed by which PABGG was coupled to the amino group of D-glucosamine with the aid of EDC. This method required a large excess of D-glucosamine in order to prevent polymerization of the PABGG. In the second method PNBGG-GLU was selectively reduced to PABGG-GLU by catalytic transfer hydrogenation with palladium on charcoal as the catalyst and sodium formate as the reductant (cf. Brieger and Nestrick, 1974; Anwer and Spatola, 1980).

The products of the two syntheses were found to be identical by several criteria, including PMR spectrum (Fig. V-2), UV absorbance spectrum (Fig. V-3), TLC mobility, elemental analysis, and optical rotation.

Azo coupling of diazotized PABGG-GLU to α -amanitin. A synthesis of ABGG-GLU was devised which provided significantly increased yield over previously reported procedures (Falck-Pederson et al., 1983; Preston et al., 1981). First, because of its low nucleophilicity, the aryl amine function of PABGG-GLU was diazotized in dilute HCl (Fig. V-1) in which the potent nitrosating agent nitrosyl chloride is generated (Challis and Butler, 1968). Second, the azo coupling reaction was conducted in a sodium phosphate buffer at pH 8.0. At this pH the phenolic hydroxyl of AMA, which has a pKa of approximately 10 (Falck-Pederson, 1981), can be expected to be slightly ionized and the tendency of the diazotized PABGG-GLU to be converted to an unreactive diazotate is slight (Zollinger, 1961). The yield of ABGG-GLU obtained using these conditions is reliably 50-60% and most of the remainder of the AMA is recovered in unreacted form.

Properties of ABGG-GLU. ABGG-GLU is in many ways very similar to its predecessor ABGG. Their UV/visible absorbance spectra (see Fig. V-4) are indistinguishable. They demonstrated very similar potency of inhibition of CT RNAP II; ABGG-GLU showed a K_i of 4×10^{-9} M, while that of ABGG was 3×10^{-9} M. Examination of ABGG-GLU by reverse phase HPLC under analytical conditions showed two poorly separated peaks. These apparently represent the two diastereomers which exist in solution as a consequence of the anomeric nature of the sugar residue. A similar phenomenon has been observed for PNBGG-GLU and PNBGG-GAL

Fig. V-2. Proton magnetic resonance spectrum of PABGG-GLU in D_2O .



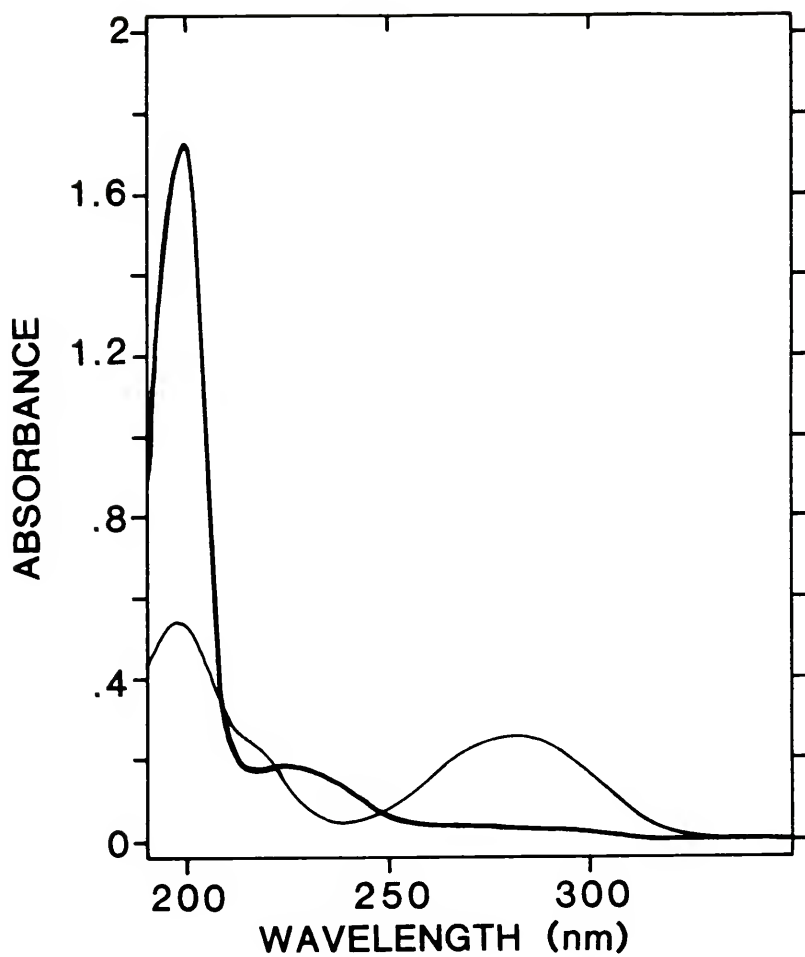
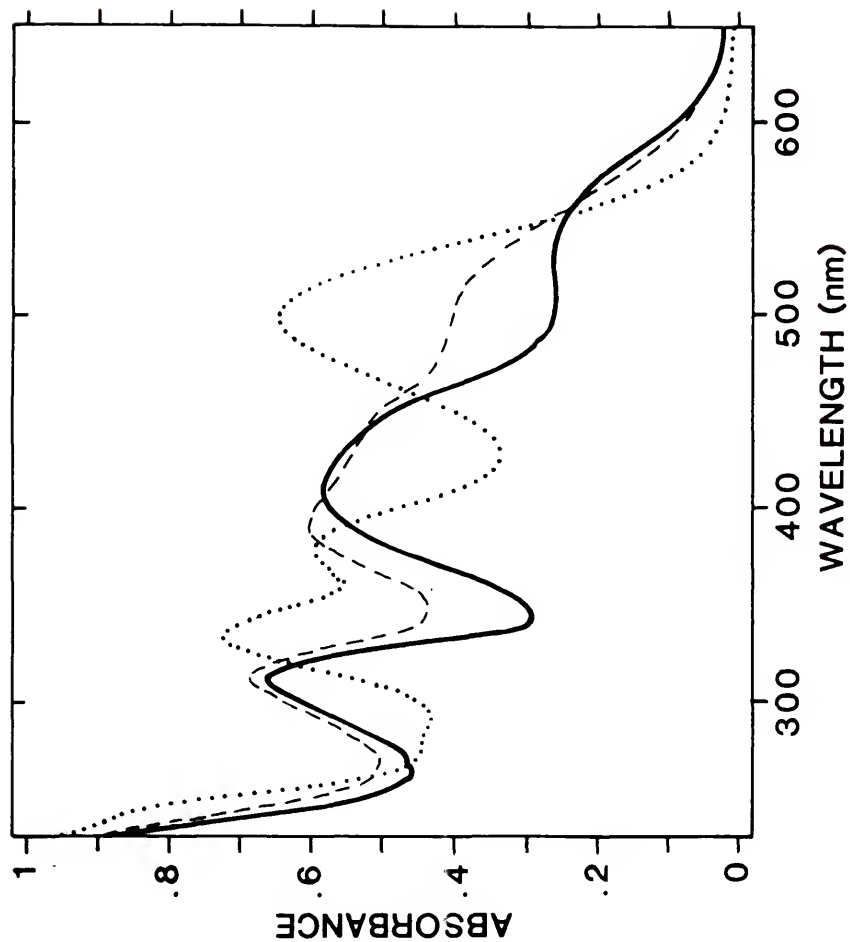


Fig. V-3. UV absorbance spectrum of PABGG-GLU at 1.8×10^{-5} M in 5 mM sodium phosphate, pH 7.0, (light line) and 0.1 M HCl (dark line).

Fig. V-4. Absorbance spectrum of ABGG-GLU at approximately 4×10^{-5} M in 5 mM sodium phosphate buffers of pH 3.0 (solid line), pH 7.0 (dashed line), and pH 11.0 (dotted line).



(data not shown). FAB-MS studies of ABGG-GLU supported the predicted structure, showing mass ions of the expected sizes (Fig. V-5).

Effects of Alkaline pH and Nickel (II) Ion on ABGG-GLU

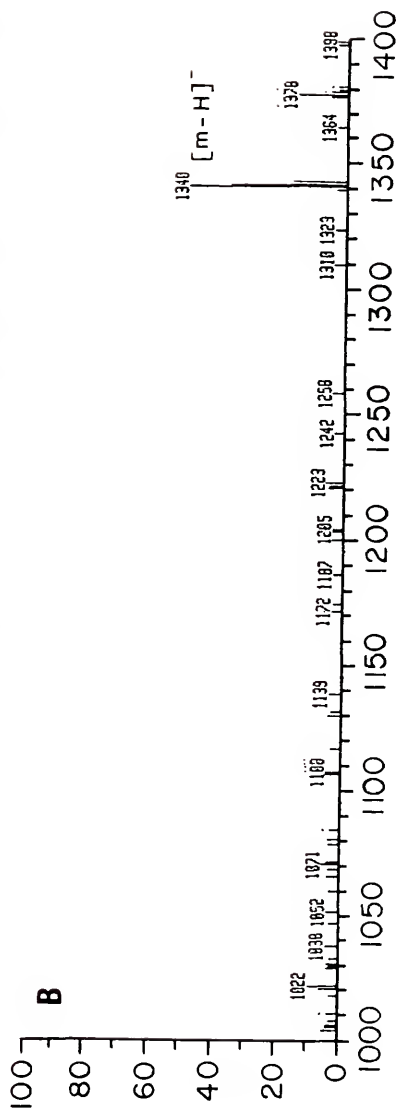
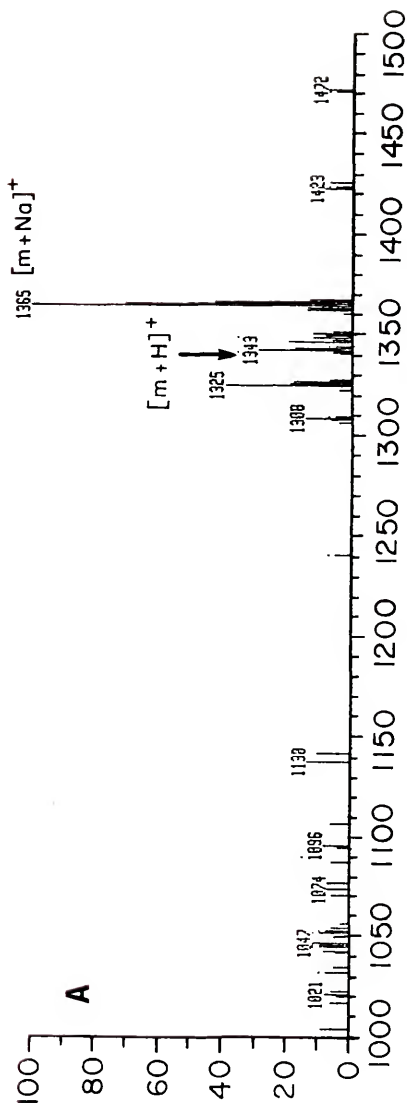
Preliminary trials of conjugation of ABGG-GLU to proteins revealed some unexpected problems. The most readily noticed of these was that the nickel (II) ion, which was added to absorb the cyanide generated in the course of the reaction, reacted with ABGG-GLU to form a complex as evidenced by an altered absorbance spectrum (Fig. V-6). ABGG-GLU could be recovered intact from the complex by adding EDTA to the solution, but at least 45 min was required for the action of the EDTA to be completed.

Also, when conjugation was undertaken at pH 8.0, decolorization of the solution was noted over a period of days. This was faster in reactions where the nickel (II) chloride was omitted. The loss of color appeared to correlate with the appearance of a new toxin-related entity which had very low mobility on TLC. When ABGG-GLU was placed at 37°C in 0.2 M sodium borate buffer, pH 8.0, a slow alteration of the UV/visible absorbance spectrum was noted (Fig. V-7).

Conjugation of ABGG-GLU to BSA

Influence of pH and reactant concentrations on the rates of toxin conjugation and alteration. Since difficulties with degradation of the toxin were noted under the conditions which were developed for coupling the PNBGG derivatives to BSA, means of circumventing this problem by altering the reaction conditions were sought. It had been noted in work with PNBGG-GLU that significant coupling occurred at

Fig. V-5. Fast atom bombardment mass spectra of A8GG-GLU in the positive ion (panel A) and negative ion (panel B) modes.



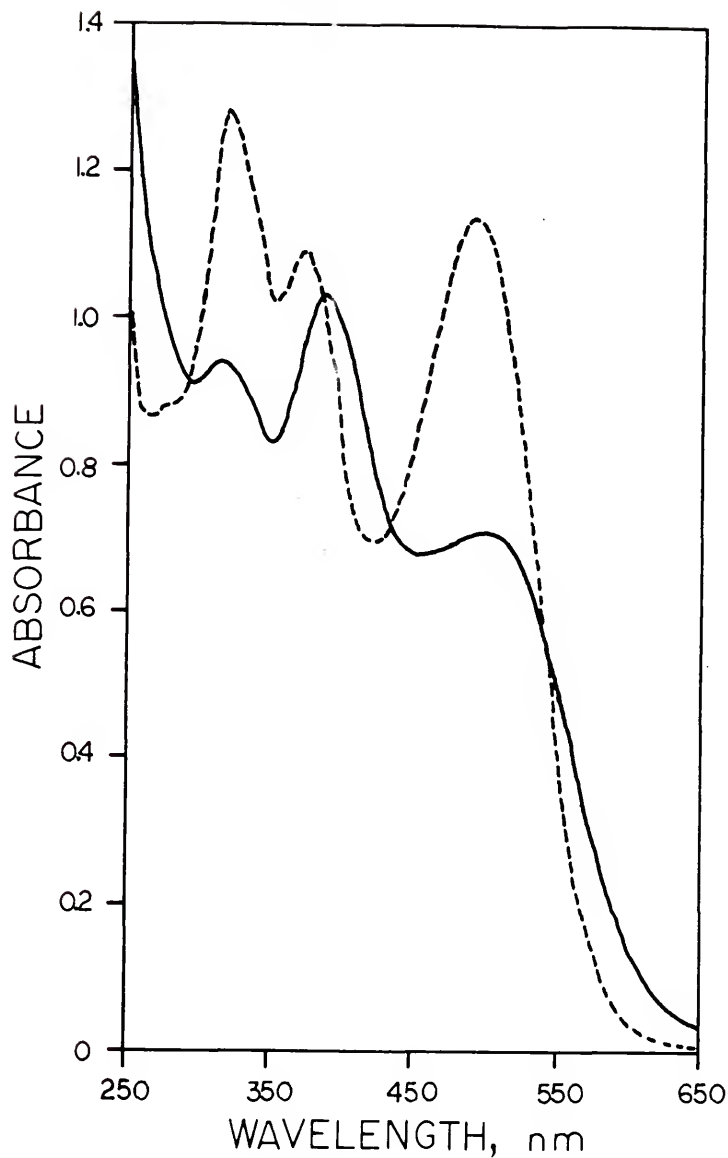


Fig. V-6. Absorbance spectrum of ABGG-GLU at 8×10^{-5} M in the presence of 10 mM nickel (II) chloride (solid line) and 45 min after the addition of EDTA (dashed line).

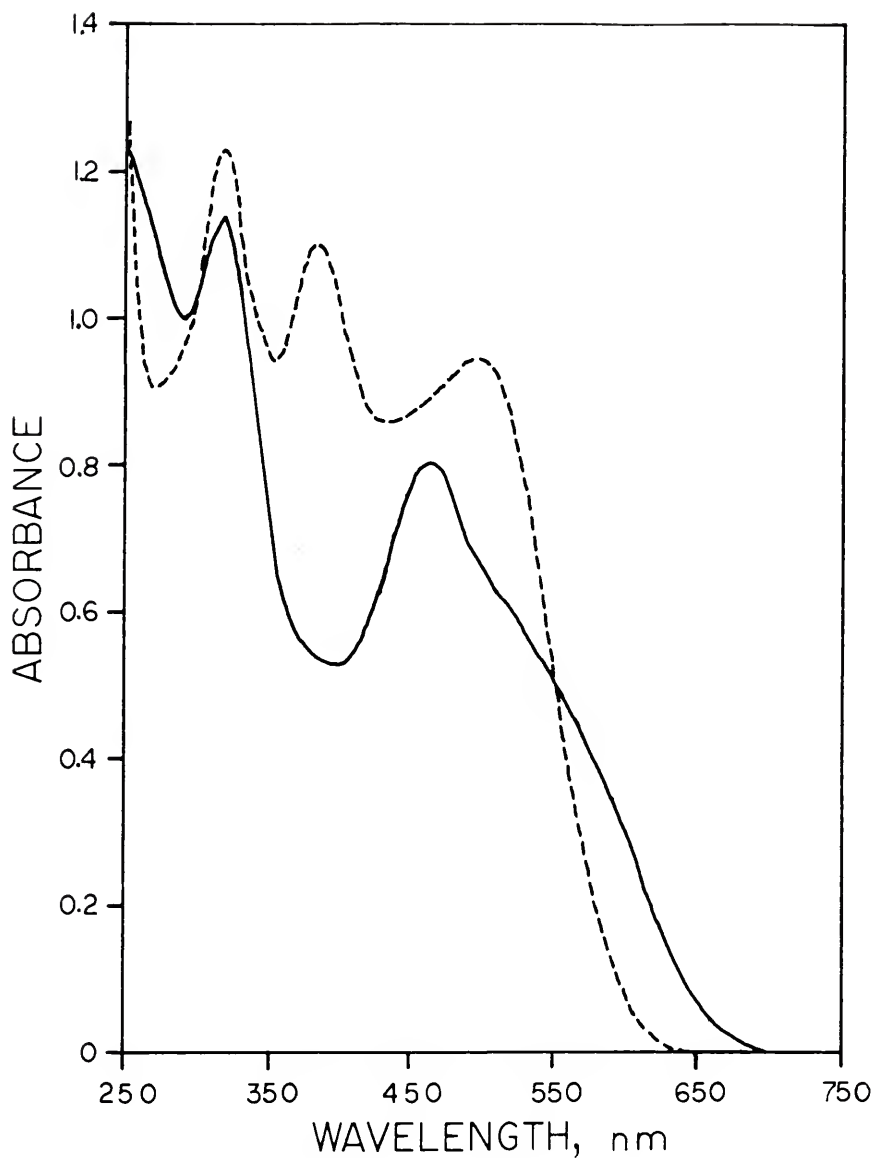


Fig. V-7. Effect of incubation of ABGG-GLU at pH 8.0 and 37°C on its absorbance spectrum. The ABGG-GLU concentration was initially 7.2×10^{-3} M and a sample was diluted 100-fold in order to obtain the spectrum. Dashed line, ABGG-GLU at pH 8.0; solid line, ABGG-GLU after 13 days at pH 8.0 and 37°C.

pH's as low as 7.0 (see chapter IV). Thus, conjugation of ABGG-GLU to BSA at pH 7.0 was compared to pH 8.0. Also, the effect of raising some of the reactant concentrations on the rates of conjugation and toxin alteration was examined.

As indicated in Table V-1, increasing the BSA and ABGG-GLU concentrations five-fold produced a three- to five-fold increase in the rate of conjugation. As expected, conjugation proceeded more rapidly at pH 8.0 than at pH 7.0; the difference in rate was most pronounced early in the reaction (after 8 hours). The rate of alteration of the toxin did not show a significant dependence on the concentration of ABGG-GLU or BSA, but was higher at pH 8.0.

Under all of these reaction conditions a single major sideproduct eluted before ABGG-GLU in the analytical reverse-phase HPLC system. ABGG-GLU eluted as two poorly resolved peaks after approximately 10 min, while the byproduct emerged as a highly symmetrical peak after about 8 min. Preparative reverse-phase HPLC of pooled, desalted ultrafiltrates from these reactions yielded a quantity of the sideproduct which was amenable to analysis. The sideproduct showed a K_i (4×10^{-9} M) with CT RNAP II and a UV/visible absorbance spectrum which were indistinguishable from those of ABGG-GLU.

Influence of nickel (II) ion, citrate, and sodium cyanoborohydride on the rate of toxin conjugation and alteration. Additional experiments were conducted to determine the importance of the nickel (II) ion and sodium cyanoborohydride on the rates of conjugation and alteration of the toxin. As noted above, a protective effect of nickel (II) ion had been detected in early studies conducted at pH 8.0. In those studies citrate was included to permit solution of the nickel

Table V-1. Effect of pH and reactant concentrations on the degradation of ABGG-GLU and on the extent of conjugation of ABGG-GLU to BSA.

concentration of BSA ^a	pH	time of reaction	ABGG-GLU/BSA ^b	% ABGG-GLU unreacted ^c
1 mg/ml	7.0	8 hours	0.30	98
1 mg/ml	8.0	8 hours	0.63	85
5 mg/ml	7.0	8 hours	1.31	107
5 mg/ml	8.0	8 hours	1.98	69
1 mg/ml	7.0	48 hours	1.06	65
1 mg/ml	8.0	48 hours	1.55	28
5 mg/ml	7.0	48 hours	4.88	59
5 mg/ml	8.0	48 hours	5.09	42

^aAll reaction mixtures contained 0.5 M sodium borate, 0.125 M PIPES, 25 mM nickel (II) chloride, and 25 mM sodium citrate. ABGG-GLU was present in a molar concentration which was 240 times that of BSA.

^bThe molar ratio of ABGG-GLU to BSA in the conjugates was determined spectrophotometrically.

^cThe amount of ABGG-GLU which remained in unreacted form in the reaction mixtures was determined by analytical HPLC.

(II) ion in the alkaline solution. The results presented in the section above suggested that the conjugation of ABGG-GLU might be more profitably conducted at pH 7.0 rather than pH 8.0. At pH 7.0 nickel (II) chloride is quite soluble without the inclusion of a chelator like citrate. Therefore, experiments were conducted to examine the need for the nickel (II) ion in the reactions at pH 7.0 and the impact of citrate upon any effect of the nickel (II) ion (Table V-2). The inclusion of citrate in the reaction did not appear to have a pronounced effect upon either the rate of conjugation or the rate of alteration of the toxin, while omission of the nickel (II) ion from the reaction resulted in an acceleration of both. When sodium cyanoborohydride, nickel (II) chloride, and citrate were all omitted from the reaction, loss of the toxin was almost negligible and coupling of the toxin to BSA occurred at a very low, but significant, level (cf. chapter IV). The conjugate prepared in the presence of nickel (II) chloride demonstrated a K_i of 1.35×10^{-7} M (with respect to amanitin residues), while that prepared without nickel was slightly less inhibitory with a K_i of 6.1×10^{-7} M.

Discussion

The azo coupling reaction has provided a relatively simple means of selectively introducing chemically complex moieties into a single site on the amanitin molecule. Thus, one can attach a complex linker and a conjugable group en bloc. Some refinements of the synthetic procedures have been introduced in this work which serve to make this route to conjugable derivatives of amanitin more attractive and practical. The nitrobenzoyl group has continued to serve as a convenient

Table V-2. Effect of nickel (II) chloride, citrate, and sodium cyanoborohydride on the degradation of ABGG-GLU and on the extent of conjugation of ABGG-GLU to BSA.

reaction components ^a	ABGG-GLU/BSA ^b	% ABGG-GLU unreacted ^c
nickel, citrate, and cyanoborohydride	3.6	72
nickel and cyanoborohydride	4.6	79
cyanoborohydride	9.6	49
no additions	0.8	96

^aAll reaction mixtures contained 5 mg/ml BSA, 18 mM ABGG-GLU, 0.5 M sodium borate, and 0.125 M PIPES. Nickel (II) chloride (25 mM), sodium citrate (25 mM), and sodium cyanoborohydride (100 mM) were also added as indicated. The reaction mixtures were incubated in the dark for 30 hours at 37°C.

^bThe molar ratio of ABGG-GLU to BSA in the conjugates was determined spectrophotometrically.

^cThe amount of ABGG-GLU which remained in unreacted form in the reaction mixtures was determined by analytical HPLC.

precursor for the necessary aryl amino function (cf. Faulstich and Trischmann, 1973; Preston et al., 1981). Catalytic transfer hydrogenation has proven to be an efficient and selective method for reducing the nitro group to an amine without need for a hydrogenation apparatus.

The reductive conjugation of aldehydes to proteins has been shown to be relatively straightforward by several workers (Jentoft and Dearborn, 1979; Schwartz and Gray, 1977; Wong et al., 1985). While the alkylation reaction has proven to be very specific for protein amino groups, some side reactions have been defined. In reductive methylation of proteins the cyanide which is generated in the course of the reaction can react with an intermediate of the reaction to form a quasi-stable adduct (Gidley and Sanders, 1982). The inclusion of a metal ion, such as the nickel (II) ion, which complexes with cyanide has been shown to be effective in overcoming this problem (Jentoft and Dearborn, 1980). In the case of reductive methylation some reduction of formaldehyde has been observed, especially at lower pH's (Jentoft and Dearborn, 1979), but there have been no comprehensive studies of the fate of sugars which were being reductively coupled to proteins.

It was hoped that the recognized side reactions due to cyanide and reduction of the aldehyde function could be avoided by including the nickel (II) ion in the reaction system and by maintaining a slightly alkaline pH. It also seemed prudent to include the nickel (II) ion in order to prevent the well-recognized degradation of protein by cyanide (Catsimpoolas and Wood, 1964; Catsimpoolas and Wood, 1966; Wood and Catsimpoolas, 1963). It was noted that the nickel (II) chloride produced a profound change in the color and absorbance spec-

trum of ABGG-GLU. This is likely due to complexation of the nickel ion by the azo amanitin. The formation of coordination complexes between transition metal ions and *o*-hydroxyazo compounds has been known for many years (Zollinger, 1961). It is conceivable that two ABGG-GLU molecules may be able to coordinate with a single nickel (II) ion.

In initial studies of reductive coupling of an azo amanitin to protein a diminution of the color of the reaction was noted after prolonged periods of reaction. This color change was most prominent in reactions to which no nickel (II) chloride was added. Since the color of the azo amanitins derives from the aromatic portion of the molecule, it was reasoned that this part was being altered. Several possible side-reactions were envisioned. One possibility was that the tryptathionine portion of the molecule was undergoing β -elimination because of the slightly alkaline pH and elevated temperature. Indeed, when ABGG-GLU was incubated at 37°C in 0.2 M sodium borate, pH 8.0, alteration of the absorbance spectrum was noted over the course of several days. It was also conceivable that the azo bridge was being reduced; the nickel (II) ion might be protective against the reduction by virtue of the formation of the coordination complex. In fact, the chemical stability of azo dyes has been noted to be increased by complexation with transition metal ions (Zollinger, 1961).

Experiments were performed in order to determine whether the rate of side reactions could be reduced relative to the rate of the conjugation reaction. Increasing the concentrations of ABGG-GLU and BSA has been found to have a positive effect in this regard; the rate of conjugation was increased without greatly effecting the rate of loss of ABGG-GLU.

The major byproduct could be purified and had the same absorbance spectrum and K_i toward CT RNAP II as ABGG-GLU. It eluted from the C18 reverse-phase HPLC system before ABGG-GLU, suggesting that it is more polar than ABGG-GLU. In contrast to ABGG-GLU which elutes as two poorly resolved peaks, the byproduct eluted as a single, sharp peak. The two components of ABGG-GLU presumably reflect the fact that the sugar residue has two anomeric configurations. Altogether, these characteristics indicate that this byproduct likely results from reduction of the aldehyde group of the sugar to a hydroxyl group. Thus, one might expect that factors which make the aldehyde group more available (e.g., increased pH) would increase the rate of formation of this byproduct. This is, in fact, the pattern which is seen.

Additional experiments were undertaken to evaluate the effect of nickel and cyanoborohydride on the course of the reaction at pH 7.0. In the absence of nickel the rates of both conjugation and loss of ABGG-GLU were substantially higher. The presence of citrate had no significant effect upon the course of the reactions which contained nickel. In the absence of cyanoborohydride there was very little conjugation of ABGG-GLU to BSA and there was no significant loss of the azo amanitin after thirty hours of reaction. This shows that both the conjugation and the side reaction(s) are strongly cyanoborohydride-dependent under these conditions. The lower conjugation and side-reaction rates observed in the presence of the nickel (II) ion probably have a complex origin, but may be largely a consequence of complexation of ABGG-GLU to the nickel. If these complexes form predominantly in a stoichiometry of two ABGG-GLU per nickel ion, then the reactivity

of ABGG-GLU may be diminished on account of its lower effective concentration.

The conjugates of ABGG-GLU and BSA prepared here proved to be relatively poor inhibitors of CT RNAP II. However, this low level of inhibitory activity of amanitin-BSA conjugates has been consistently observed by other workers. The significance of a high or low K_i for the interaction between RNAP II and an amanitin-protein conjugate is not at all clear. As of yet, the molecular determinants of these K_i 's are not known. Despite their high K_i 's, amanitin-BSA conjugates have in the past proven to be highly cytotoxic to cells in culture (Faulstich et al., 1975; Hencin and Preston, 1979; Preston et al., 1981).

In summary, an efficient method of preparing an azo derivative of AMA has been described. Reaction conditions for the coupling of this azo derivative to BSA have been optimized so that substantial amounts of the toxin can be linked to the protein within a reasonable time. The conjugates thus produced have inhibitory properties toward CT RNAP II which are similar to those reported by others. The task of determining whether this new method of conjugation can be used to prepare amanitin-antibody (or SMWCA-antibody) conjugates which are as good as or better than those being generated by current methods lies ahead.

CHAPTER VI

CONCLUSIONS

Two routes for the preparation of aldehyde-containing amanitins have been explored. Study of the periodate oxidation products of OMA has provided new insights into the nature of OMAA. Under certain circumstances, a true aldehyde can be generated by periodate oxidation of OMA, but the form of OMAA which has been prepared by others appears to exist almost exclusively in a non-aldehydic form. Apparently as a consequence of this, the reductive amination of OMAA is difficult. Despite these difficulties, a number of new derivatives of amanitin have been prepared which bear conservative changes in the sidechain of residue 3. A large range of inhibitory potency has been discovered for derivatives with relatively small differences in this sidechain. A preliminary correlation between the presence of a charged group in residue 3 and markedly diminished inhibitory potential has been noted.

An efficient method for preparing an azo amanitin which bears a residue of glucosamine has been described. The glucosamine residue has been found to share with lactose a strong tendency to expose its aldehyde function in solutions of borate, as evidenced by increased reactivity in reductive alkylation reactions in this medium. This property has been utilized for the preparation of an azo amanitin which bears an aldehyde which is protected within the sugar; it can be selectively deprotected in solutions of borate to permit efficient reductive coupling of the amanitin to proteins.

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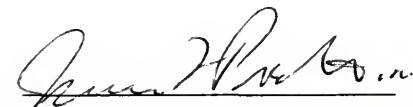
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BIOGRAPHICAL SKETCH

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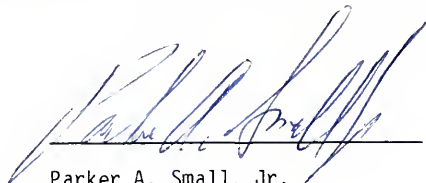
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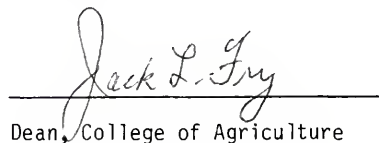
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